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INFLUENCE OF CHEMICAL COMPOSITION OF ORGANIC WASTE ON BIOLOGICAL HYDROGEN PRODUCTION

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CONTENTS

PREFACE

My master degree experience was in Environmental Engineering at the University of Padova. During this period my interests focused on solid waste management and especially on renewable energy production. In this historical period environmental problems increase rapidly and I became sensitive to topics like world energy demand, waste production and air, water and soil pollution. In my thesis work I decided to go deeper inside the anaerobic digestion process, that touch and try to solve all the above mentioned problems. In addition to this, I was really interested in having a practical experience and a direct involvement on scientific experiments and chemical analysis. Prof. Raffaello Cossu, my supervisor, gave me the possibility to conduct my thesis in the Environmental and Sanitary Engineering Laboratory of the ICEA Department of the University of Padova, located in Voltabarozzo. Then Dr. Luca Alibardi, my co-supervisor, proposed me to study the hydrogen production process from the organic fraction of municipal solid waste. The objectives of the research were to simulate the OFMSW with food products to have a reproducible substrate and to use it in order to analyze the influence of the chemical composition of substrate, in terms of carbohydrates, proteins and lipids content, on hydrogen production.

My experience started in September 2013. In the first month I focused my attention on bibliographic research and reading of scientific papers on the topic of my research. At the same time I started looking for information about chemical composition of different food products and I analyzed historical data of real OFMSW production and composition of previous thesis works of students Paolo Armaroli and Alessandra Ruzza realized in the same laboratory as me. Finally specific food products were chosen to represent the single categories of OFMSW. In particular, raw chicken breast, tuna and butter were selected for 'Meat, Fish and Cheese' category, apple-banana mousse for 'Fruit', lyophilized minestrone soup for 'Vegetable' and breadcrumbs and raw pasta for 'Bread and Pasta'.

The first part of BHP (Biochemical Hydrogen Potential) tests were conducted in batch reactors on the four single categories above mentioned using two different types of sludge, an anaerobic sludge coming from an anaerobic digester and a granular sludge collected from a full-scale Upflow Anaerobic Sludge Blanket (UASB) anaerobic digester. Dr. Annalisa Sandon, the chemical technician of the laboratory, taught me how to do the analysis to characterize both substrate and sludge. TS, VS and TKN analysis were performed. Moreover, during BHP tests, the amount of gas produced was measured and samples were taken and analyzed through a gas chromatograph for gas quality in terms of H_2 and CO_2 concentrations. In addition liquid samples

were collected at the end of the tests, filtered and analyzed for DOC, N as NH_4^+ and VFAs concentrations. pH was also monitored.

After this, 8-different mixtures were defined to study the influence of chemical composition of substrate on hydrogen production. The mixtures were prepared using the same food products utilized to simulate OFMSW categories. Exact % of carbohydrates, proteins and lipids were chosen for each mixture. In four mixtures the % of carbohydrates was reduced from 65% to 35% and the one of lipids consequently augmented from 20% to 50% (10% intervals) maintaining the amount of proteins constant to the value of 15%. The same was done for other four mixtures in which lipids were maintained constant to 15%, carbohydrates reduced from 65% to 35% and proteins augmented from 20% to 50%. A mathematical model was implemented to determine the correct raw weight percentage of each food products in every mixture.

The second part of BHP tests were, then, conducted on the 8-different mixtures in batch reactors utilizing the same two types of sludge used in BHP tests on single categories. Mixtures were analyzed for TS, VS, TKN and TOC. BHP tests were performed in the same way described before.

After this second phase of experiments, I concentrated myself in collecting and elaborating all data obtained till that moment. Chemical composition showed to have an important role in hydrogen production.

Finally, the third part of BHP tests were conducted on four selected mixtures utilizing a batch stirred reactor. The aim was to confirm previous results obtained in simple batch reactors and better analyze the hydrogen production process. Indeed in these experiments it was possible to register data about biogas production every ten minutes, allowing the determination of a very precise curve of gas generation in time. Data were also interpolated using the Gompertz equation. Anaerobic sludge was used in these types of tests and the chosen mixtures were the ones with higher content of carbohydrates (65%), lipids (50%) and proteins (50%) to better analyze the influences of these chemical compounds in the biological hydrogen fermentation. Two pH conditions were tested, 5.5 and 7.0. Moreover, COD analyses were performed on solid and liquid samples. Gas and liquid samples were collected and analyzed in the same way as for the other BHP tests.

The thesis activity in the laboratory was concluded in March 2014 and the experience widely satisfied the initial expectations. The results collected enable a better understanding of biological hydrogen fermentation process and the influence on it of the chemical composition of the substrate in terms of carbohydrates, proteins and lipids content.

I want here to take some space to thank Prof. Raffaello Cossu, Dr. Luca Alibardi, Dr. Annalisa Sandon and the whole staff of Voltabarozzo to help me and follow me in my thesis work; thanks to my family and my friends to be close to me in this important step of my life.

1. INTRODUCTION

Nowadays, the three big problems regarding the conditions of the environment are the increasing world energy demand and waste production, mainly due to population growth and progressive industrialization, and the strictly connected air, water and soil pollution that gives rise to many human health diseases.

The International Energy Agency (IEA) has predicted an increase by more than 50% until 2030 in global demand for energy (Ball and Wietschel, 2009). Moreover, about 80% of the total energy is now produced exploiting fossil fuels that are a non-renewable energy source going under depletion and which combustion leads to the release to the atmosphere of pollutants, like CO_x, NO_x, SO_x, C_xH_x and others, that cause global climate change and health problems (Das and Veziroglu, 2001).

To face this scenario European Community has set specific constrains in European Union (EU) legislative framework on energy production from renewable resources, maximization of materials recycling and landfilling of biodegradable waste (De Gioannis *et al.*, 2013). According to this, hydrogen and methane production from two-stage anaerobic digestion process of biological residues can be considered a good solution. The process can produce energy rich gases from the organic fraction of Municipal Solid Waste (MSW) or other residues from industrial processes (like agricultural and food industry, breeding farm, wastewater treatment plan etc.) and in the same time treat these materials in order to get a strong reduction of biodegradable material content. In this way energy is produced from waste, a renewable resource, that are at the same time stabilized, reduced in volume and potentially being further available, after appropriate aerobic treatment, to be used as compost for land applications and so recycled.

Hydrogen is a secondary energy source, like electricity, and this means that it is produced from any available primary energy source. Hydrogen is the lightest element and most abundant in the universe and it is available on earth only in compounds. Its calorific value per unit weight is 142 MJ/kg being the highest above common fuels as methane (55 MJ/kg), petroleum (43 MJ/kg), coal (15-27 MJ/kg), dry wood (14-17 MJ/kg). It is environmentally and climatically clean at its point-of-use, as it is emission-free (only water is emitted from combustion with oxygen). On the other hand this characteristic is not always verified taking into consideration its production: it really depends on how it is obtained. Hydrogen can be considered a clean energy source over its entire energy conversion chain (production, storage, transport, dissemination, utilization) in the sole cases of production from renewable electricity or from fossil fuels when carbon capture and storage (CCS) is included (Winter, 2009). Moreover, it is inherently securely

safe because hydrogen energy is without radiotoxicities or radioactivity and no accidents which was causally introduced by hydrogen have been reported yet (Winter, 2009).

At present hydrogen is produced mainly from fossil fuels (steam reforming of natural gas; thermal cracking of natural gas; partial oxidation of heavier than naphtha hydrocarbons; coal gasification), biomass (pyrolysis or gasification) and water (electrolysis; photolysis; thermochemical process; direct thermal decomposition or thermolysis; biological production) (Das and Veziroglu, 2001). Global hydrogen production today amounts to around 700 billion Nm^3 and is based almost exclusively on fossil fuels: roughly half on natural gas and close to one third on crude oil fractions in refineries (Ball and Wietschel, 2009). Presently three main technologies are proven and applied on industrial scale for hydrogen production: natural gas reforming (steam methane reforming - SMR), coal gasification and water electrolysis (efficiency with current technologies is only about 65% (Hallenbeck, 2009)). The first one is considered to be the cheapest, at current feedstock prices, while the last one the more expensive; in addition the first two methods need a CCS system to face CO_2 emission problems (Ball and Wietschel, 2009). Renewable hydrogen can be obtained via electrolysis from wind or solar-generated electricity. Biomass gasification is still at an early stage, while photolysis and biological production processes are at level of basic research (Ball and Wietschel, 2009).

Most of hydrogen is produced on-site for captive use, especially as a reactant in the chemical and petroleum industries: ammonia production has a share of around 50%, followed by crude oil processing with slightly less than 40% (Ball and Wietschel, 2009). Worldwide, the amount of captive hydrogen is about seven times that of merchant hydrogen, the latter consists of gaseous and liquid hydrogen and the gaseous type is about six time the liquid one. Major hydrogen users are the space flight business and the electronics industry, glass and food manufactures and electrical equipment companies (Winter, 2009). A future challenge is to take advantage of hydrogen as mobile fuel source implementing H_2-fueled fuel cell vehicles: today, the efficiency of the fuel cell system for passenger cars is around 40% (in the future maybe 50%) compared to 25-30% for gasoline/diesel powered internal combustion engine under real driving conditions (Ball and Wietschel, 2009). Hydrogen-fueled fuel cells are compact, quiet, clean and highly efficient (Winter, 2009) but improvements are still needed to gain cost reduction. In combustion, water is the main product, thus, H_2 is regarded as clean non-polluting fuel. Finally, hydrogen could further be used as a storage medium for electricity from intermittent renewable energies such as wind power (Ball and Wietschel, 2009).

Biological hydrogen production processes are less energy intensive if compared to previous processes due to the fact that operate at ambient temperatures and pressures. Different processes

exist: biophotolysis of water by green algae (direct) and by cyanobacteria (indirect); biological water-gas shift reaction; photo-fermentation of organic compounds by photosynthetic bacteria; dark-fermentation from organic compounds by strict or facultative anaerobic bacteria (Das and Veziroglu, 2009; Ni *et al.*, 2006). Conversion efficiencies for direct biophotolysis are below 1% and indirect biophotolysis remains to be demonstrated (Hallenbeck and Benemann, 2002). Photodecomposition method has been extensively studied and is the most used till today: it is a theoretically perfect process with transforming solar energy into hydrogen by photosynthetic bacteria, but applying it to practice is difficult due to the low utilization efficiencies of light and difficulties in designing the reactors for hydrogen production. However, fermentative hydrogen production has the advantages of rapid hydrogen production rate, simple operation, constant production through day and night and utilization of various organic waste as substrate. Finally, it is more feasible and thus widely used than the photosynthetic process (Wang and Wan, 2009). Unlike a biophotolysis process that produces only H_2, the products of dark fermentation are mostly H_2 and CO_2 combined with other gases, such as CH_4 or H_2S, depending on the reaction process and the substrate used (Ni *et al.*, 2006). All biological processes mentioned are controlled by the hydrogen-producing enzymes, such as hydrogenase and nitrogenase. Nitrogenase has the ability to use magnesium adenosine triphosphate (MgATP) and electrons to reduce a variety of substrates (including protons). This chemical reaction yields hydrogen production by nitrogenase-based system:

$$2e^- + 2H^+ + 4ATP \rightarrow H_2 + 4ADP + 4Pi \qquad (1)$$

where ADP and Pi refer to adenosine diphosphate and inorganic phosphate, respectively. Hydrogenases exist in most of the photosynthetic microorganisms and they can be classified into two categories: uptake hydrogenase and reversible hydrogenase. Uptake hydrogenase, such as NiFe hydrogenases and NiFeSe hydrogenases, act as important catalysts for hydrogen consumption as follows:

$$H_2 \rightarrow 2e^- + 2H^+. \qquad (2)$$

Reversible hydrogenases, as indicated by its name, have the ability to produce H_2 as well as consume H_2 depending on the reaction condition (Ni *et al.*, 2006).

Dark-fermentative hydrogen production represents one part of the whole process of anaerobic digestion (AD) of biodegradable organic substances. The AD process consists of four main steps:

hydrolysis to soluble products; conversion of monomers to volatile fatty acids (VFAs) and alcohols by acidogenic bacteria (acidogenesis); conversion of propionic, butyric and alcohols to acetate, CO_2 and H_2 by acetogenic bacteria (acetogenesis); and final conversion of acetate and hydrogen to methane (methanogenesis) (Trzcinski and Stuckey, 2012). This is also called one-stage process and leads to direct CH_4 production, that can be used for heat and power co-generation.

On the other hand, it is possible to split the above mentioned anaerobic digestion process in a two-stage system separating the acetogenic and methanogenic phases. Two sequential separated reactors are provided: in the first hydrogen and carbon dioxide are the gaseous products and VFAs are released into the liquid solution, while in the second one final conversion of the residual biodegradable organic matter into methane and carbon dioxide is achieved. A great number of advantages has been highlighted from different authors. First of all acidogens are the fastest to grow microorganisms in AD while methanogens the most sensitive to pH variation, so phase separation avoids the suppression of methanogenic activities and possible process failure due to accumulation of VFAs and pH decrease (Elbeshbishy and Nakhla, 2012). In this way the first system could be run at more acidic pH conditions and relatively short Hydraulic Retention Time (HRT), while the second one at more basic pH and longer HRT (Hallenbeck, 2009); this also increases tolerance to high Organic Loading Rate (OLR). Secondly, it has been reported that an improved acidogenic phase results in enhanced final biogas yield (De Gioannis *et al.*, 2013); one reason could be the first stage higher solubilisation. In addition, according to Hallenbeck (2009), the combination of the two gas streams would create a hydrogen-methane mixture (~20-30% H_2, after removal of CO_2, and 80-70% CH_4) showing to burn cleaner than methane alone. Moreover, as already stated, H_2 has the higher calorific value per unit weight of any known fuel. On the other hand, two main disadvantages can be stressed out: the increase of operational costs splitting the process in a two-stage system and the inadequate technologies in hydrogen exploitation at present situation (De Gioannis *et al.*, 2013; Ball and Wietschel, 2009; Winter, 2009).

The relevant steps of the biological process have been described above; the following chemical reactions depict the different metabolic pathways of H_2 production and depletion (Guo *et al.*, 2010; De Gioannis *et al*, 2013.). Hydrogen production includes acetate and butyrate pathway (equations (3) and (4), respectively) and other forms of degradation of the same compounds (equations (5) and (6)).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2CH_3COOH \qquad (3)$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + CH_3CH_2CH_2COOH \quad (4)$$

$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2H_2 + 2CH_3COOH \quad (5)$$

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (6)$$

On the other hand, other reactions can take place in the system leading to the formation of propionic acid, ethanol or also acetate, in which hydrogen is consumed (equations (7), (8) and (9), respectively). In addition, zero-hydrogen production pathway is also possible, with formation of ethanol or lactic acid (equations (10) and (11), respectively).

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (7)$$

$$CH_3COOH + H_2 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (8)$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (9)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (10)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \quad (11)$$

In mixed cultures, a ratio of 3:2 of butyrate/acetate is usually observed, originating from the combination of equations (3) and (4):

$$4C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 3CH_3CH_2CH_2COOH + 8CO_2 + 10H_2 \quad (12)$$

The major H_2-producing bacteria are related to strict anaerobic genera (*Clostridia*, methylotrophs, rumen bacteria, methanogenic bacteria, archaea), to facultative anaerobic genera (*Escherichia Coli*, *Enterobacter*, *Citrobacter*) and to aerobic genera (*Alcaligenes*, *Bacillus*) (Guo *et al.*, 2010).

It is important to point out that numerous parameters influence dark-fermentation process. A brief list of them includes: substrate types, co-digestion of substrates and relative ratio, inoculum type and origin, food/microorganism (F/M) ratios, applied pre-treatment to substrate and inoculum, reactor configuration, temperature, pH, nitrogen, phosphate and metal ion availability, OLR, HRT and gas partial pressure (Wang and Wan, 2009; De Gioannis *et al.*, 2013; Ni *et al.*, 2006). These factors greatly affect hydrogen fermentation yields and kinetics and many different experiments have been conducted at lab scale to evaluate their effects. Indeed no data on full-scale hydrogen fermentation plants are currently available and only some experiences have recently been gained on pilot-scale reactors (De Gioannis *et al.*, 2013). Each parameter will be shortly described through an analysis made on scientific material illustrating laboratory experiments.

The first factor that is taken in consideration is the type of substrate utilized in the fermentation. Large experiences have been conducted on glucose, sucrose and starch, but even complex substances could be suitable for bio-hydrogen production by dark fermentation. For example residual materials could be used, as organic fraction of municipal solid waste (OFMSW) and food waste. They particularly fit the purpose due to their high carbohydrate content, wide availability and cheapness (De Gioannis *et al.*, 2013). Moreover, these types of substrate could be mixed with other types, like agricultural, farm and industrial waste (mainly sludge from wastewater treatment plants), that might not be indicated to be easily degraded as sole-substrate. In addition, co-digestion could be advantageous in having internal control of pH and optimization of the carbohydrate to proteins ratio, due to the characteristic of proteins to be a source of nitrogen for biomass growth and of alkalinity. Different values of optimal substrate concentration have been tested in many studies as reported by the review of Wang and Wan (2009) on factors influencing fermentative hydrogen production.

Secondly, the type of inoculum is another important element that has to be evaluated. Various pure cultures or mixed microbial cultures have been tested. The second type seems to be preferred because the system would be cheaper to operate, easier to control and capable of digesting a variety of feedstock materials. Some examples are: anaerobic sludge from full-scale anaerobic digesters, granular sludge from UASB (Upflow Anaerobic Sludge Blanket), waste activated sludge, cattle manure, compost and others (De Gioannis *et al.*, 2013; Wang and Wan, 2009). However, in natural environment (like sludge), the problem of coexistence of H_2-producing and consuming bacteria arises. To overcome this drawback, several pre-treatment methods have been established: heat-shock treatment (HST), acid, base, aeration, freezing and thawing and addition of specific chemical compounds. HST is the most common, the temperature is around 100°C and duration in the range of 15-120 minutes. The aim is to harvest H_2 producers, on account of their larger chance to survive when a mixed culture is treated by harsh conditions due to their ability to sporulate as a reaction to adverse environmental conditions (De Gioannis *et al.*, 2013; Wang and Wan, 2009; Kvesitadze *et al.*, 2012). Some experiments have also been conducted without inoculum, considering that mixed anaerobic consortium is already present in substrate as OFMSW.

Speaking about reactor configurations, the greater part used in laboratory consists of small-scale (100-500 ml) vessels or stirred fermenters of 2-10 l, operated under batch, semi-continuous or continuous conditions. Range of HRT of 21 h - 4 d has been reported for stirred reactors with continuous or semi-continuous operation (De Gioannis *et al.*, 2013). Most reactors operate with no biomass recycle, so HRT and Sludge Retention Time (SRT) coincide. Long SRTs favor the

buildup of H_2 consumers (methanogens) and competitors for substrates (non-H_2-producing acidogens); but low SRT may reduce the substrate utilization efficiency. Considering OLR, it affects VFA accumulation, pH changes (which is a function of system's alkalinity) and variation in the composition of the active biomass, with consequent modification of the associated metabolic pathway. Comparison of different studies is difficult and these ranges have been found: 8-38 kgVS/(m^3·d) or 20-64 kgCOD/(m^3·d) (De Gioannis *et al.*, 2013). At large-scale operations continuous production processes would be required and other reactors types could be continuous stirred tank reactor (CSTR), packed bed reactor (PBR), anaerobic sequencing batch reactor (SBR), UASB.

Other two important parameters that have great influence on fermentation are temperature and pH. Most of experiments are run under mesophilic conditions (30-45°C, typically 35-37 °C), but also termophilic conditions are possible (50-60 °C). Temperature has an important role in dictating the nature of microbial consortium during the process and this has effects on production yields, higher at 50°C (De Gioannis et al., 2013). Nevertheless, higher energy consumption at termophilic conditions has to be taken into account.

In general, pH is considered the most pivotal parameter due to its effects on hydrogenase activity, metabolic pathways and substrate hydrolysis. It could be set at specific initial values, normally in the range 5-9, and/or controlled along the process, within values of 5 and 7 (most commonly 5-5.5) (De Gioannis *et al.*, 2013). Ni *et al.* (2006) and Lay and Fan (2003) reported optimal pH values between 5 and 6. Acetate and butyrate production have been reported to be favored in the pH range 4.5-6.0, while neutral or higher pHs are believed to promote ethanol and propionate production (H_2-consuming pathway).

Finally, it is important that right content of essential nutrients, like nitrogen and phosphorous, and micronutrients, as trace level of metal ions, is present for hydrogen-producing bacteria growth. Wang and Wan (2009) reported different values from different studies for optimal C/N and C/P ratio: 200 and 74 for the first, and 1000 and 559 for the second one. Several studies also investigated the toxicity of heavy metals.

To improve H_2 production, some manipulations have been proposed, as decreasing H_2 partial pressure using inert gas sparging, or CO_2 removal from culture liquid (Hallenbeck, 2009; Das and Veziroglu, 2001). Ni *et al.* (2006) explains that when H_2 concentration increases, the metabolic pathways shift to produce more reduced substrates, such as lactate, ethanol, acetone, butanol or alanine, which in turn decrease the H_2 production.

Table 1 illustrates hydrogen yields obtained in different studies and the various conditions of the experimentations (substrate, reactor type, inoculum, temperature, pH, HRT).

Table 1. H_2 yield from different types of organic substrates at different operating conditions reported in scientific literature.

Reference	Substrate	Reactor	Inoculum, treatment	Yield	pH	HRT	T (°C)	Note
Liu et al., 2006	Household solid waste (HSW)	Continuous system	From biogas plant. 100°C, 1h	43 mlH2/gVS	5.0-5.5	2 d	37	Sparging with CH_4 (double production)
Giordano et al., 2011	Glucose, potato waste, wheatfeed	Batch conditions	Granular sludge. 105°C, 4h	185±13 mlH2/gCODadd glucose 153-186 mlH2/gVS potato 54-91 mlH2/gVS wheat	7.0	7 d	35	
Nasr et al., 2012	Thin stillage (65% carbohydrate on dry mass)	Batch, stirred 180 rpm	Acclimatized anaerobic digester sludge. Heat pretreated	247-557 mlH2/gCODrem	control 5.47	4 d	37	F/M: 4, 6 (best), 8
Kvesitadze et al., 2012	OFMSW (35-37% lignocellulosic material)	Batch, stirred 50 rpm	*Clostridia* sp	82,5-104 mlH2/gVS	9.0	14 h	55	
Ueno et al., 2007	Artificial organic solid waste	Continuous flow reactor (50 d)	Hydrogenogenic microflora	0,1-199 mmolH2/l_reactor/d	6.0-7.0	0,5-4 d		
Lee and Chung, 2010	Food waste	Pilot scale, continuous system	From anaerobic digester. 80°C, 20 min	1,82 molH2/mol_glucose	5.5	21-66 h	30	
Nathao et al., 2013	Synthetic food waste (65% rice, 17% vegetable, 18% meat)	Batch, stirred 150 rpm	From UASB. 90°C, 30 min	55 mlH2/gVS	6.0	2 d	37	F/M: 2,5-10 (7,5 best)

Chu et al., 2012	Food waste (potato; kitchen garbage; bean curd manufacturing waste)	CSTR	Anaerobic digester sludge. 70°C, 30 min	85 mlH2/gVSadd potato 66 mlH2/gVSadd garbage waste 20 mlH2/gVSadd okara-soia	5.5	2 d	55	Analysis on carbohydrates, proteins, lipids.
Okamoto et al., 2000	Simulated OFMSW (rice; cabbage; carrot; egg; lean meat; fat; chicken skin)	Batch, stirred 5 rpm	Anaerobic digested sludge. Boiled 15 min	72,6 mlH2/gVS carrot 9,75 mlH2/gVS fat 2,47 mlH2/gVS lean meat	7.0	50-200 h	37	Measure VFA and solvents
Dong et al., 2011	Simulated OFMWS (rice; potato; lettuce; lean meat; peanut oil; banyan leaves)	Batch	From swine manure anaerobic digester. Boiled 15 min	125 mlH2/gVS rice 103 mlH2/gVS potato 35 mlH2/gVS lettuce 0 mlH2/gVS lean meat 5 mlH2/gVS peanut oil 0 mlH2/gVS banyan leaves	5.5	0-7 d	37	Measure VFA and alcohols. C/N: 48 rice; 35 potato; 13 lettuce; 4 lean meat; 6967 peanut oil; 126 banyan leaves.
Kobayashi et al., 2012	MSW (20 types)	Batch, stirred 80 rmp	Digested sludge	Higher for carbohydrates	6.0	15 d	55	F/M: 1. Analysis on carbohydrates, proteins, lipids.
Boni et al., 2013	Food waste + SHW (slaughterhouse)	Batch, stirred	Activated aerobic sludge. 100°C, 30 min	145 mlH2/gVS (40%FW-60%SHW) 70 mlH2/gVS (100% FW)	5.0-6.0	5 d	36	Measue VFA. C/N: 22 FW; 3,8 SHW.
Bai et al., 2004	Glucose/starch + peptone	Batch, stirred 100 rpm	From UASB. Boiled 30 min	6,4 mmolH2/gCOD (60%glucose-40%peptone) 4,5 mmleH2/gCOD (80%starch-20% peptone)			35	Measure VFA and N conversion.

A wide range of variation is observed. Individual parameters as well as the existence of mutual interactions between them have a strong influence on process performances and can lead to variations up to three order of magnitude depending on the specific combination of the operating variables adopted. To this end, it is advisable that the scientific community makes an effort to harmonize the measurement units and the description methods utilized; this could facilitate the comparison of results from different authors (De Gioannis *et al*., 2013). In addition, it is important to highlight that often the composition and chemical nature of substrates tested is not specified.

As already said, carbohydrate-rich substances show the greater potential for H_2 production. Lay and Fan (2003), testing high-solid organic waste (HSOW) under mesophilic conditions, obtained that H_2-producing potential of carbohydrate-rich HSOW (rice and potato) was approximately 20 times larger (600ml) than that of fat-rich HSOW (fat meat and chicken skin) and of protein-rich HSOW (egg and lean meat). So it could be significant knowing the carbohydrates, proteins and lipids content of the substrate to better understand the results of the experiments. Some authors go deeper in the analysis of specific food or categories of OFMWS (Table 1), but still few correlations exist between substrate chemical composition (in term of carbohydrates, proteins and lipids) and hydrogen production. This could be the key to better understand the biological and chemical reactions behind the fermentative process, explain the different parameters influence and harmonize inconsistent results.

Hallenbeck (2009), Lay and Fan (2003) and Elbeshbishy and Nakhla (2012) explained the hydrolysis process of carbohydrates, proteins and lipids. Carbohydrates are easily and rapidly hydrolyzed by enzymes to sugars, which are then degraded by acidogens to VFAs, prior to further conversion by acetogens to acetate, CO_2 and H_2. Proteins are firstly hydrolyzed by proteolytic enzymes to peptides and amino acids; latter are principally fermented in pairs by so-called Strickland reactions where one amino acid serves as electron acceptor for the oxidation of the second one; these reactions thus yield no hydrogen. The products of fermentation are VFA, CO_2, NH_4^+ and S_2^-, as well as little H. Lipids are hydrolyzed to glycerol and long-chain fatty acids (LCFAs). LCFAs are degraded to acetate and hydrogen in natural system by syntrophic bacteria, but this reaction is only possible at extremely low H_2 partial pressure maintained by the associated methanogenic or sulphate-reducing bacteria. Lay and Fan (2003) report that even if egg (protein-rich) and rice (carbohydrate-rich) have almost the same C/H ratio (around 9), they have really different N/H ratio (egg: 1,65; rice: 0,28), that explains H_2 different yields because in egg most H combines with N as ammonium. Moreover, Elbeshbishy and Nakhla (2012) highlighted the importance to have buffering capacity in the system, so products that will

counteract the effects of the VFAs need also to be formed. To this end, carbohydrate-rich substrates are known to be good producers of VFAs, while protein-rich substrates to yield good buffering capacity due to the production of ammonia. Finally, it is important to remember that hydrolysis of proteins is slower than that of carbohydrates (Elbeshbishy and Nakhla, 2012).

In this context, the aims of this research study are the followings:

1. Evaluate the temporal variability of the OFMSW in terms of waste composition and physical-chemical characteristics. OFMSW is composed of different sub-fractions of waste products (residue of fruit, vegetable, bread-pasta, meat-cheese-fish) having different chemical composition and physical characteristics. These differences can affect hydrogen potential productions of the mixture of organic waste in MSW.
2. Evaluate how the chemical composition of the OFMSW in terms of carbohydrate, protein and lipid content, is related to the hydrogen potential productions obtained from a biological fermentative process.
3. Analyze the effects of waste composition and chemical characteristics on hydrolysis and fermentation rates.

2. MATERIALS AND METHODS

2.1 Substrate

Food products were used to simulate the organic fraction of municipal solid waste with the aim of using a reproducible substrate similar to organic waste.

Four different sub-fractions of the OFMSW were established: meet, fish and cheese; fruit; vegetable; bread and pasta. These four sub-fractions are characterized by different contents of carbohydrates, proteins and lipids and therefore differently contribute to the chemical composition of the OFMSW. One or more food products were chosen to represent each of them as reported in Table 2.

The food products were used to simulate eight different mixtures of OFMSW characterized by different percentages of carbohydrates, proteins and lipids. The specific characteristics of each mixture are reported in Table 3. Knowing the chemical composition in terms of carbohydrate, protein and lipid contents of the food products (Table 4), the amount of any food product was calculated to have the final characteristic of the mixture reported in Table 3. Mixture composition is reported in Table 5.

A mathematical model was developed to obtain the weight percentages of all 8 mixtures. The imposed data were:

- fixed % of carbohydrates, proteins and lipids of 8-mixtures (Table 3);
- food labels data of each product as g/100g_edible part (Table 4);
- 38% of VS on raw basis (assumption made on historical OFMSW data analysis);
- equal weight percentage on raw basis for tuna and raw chicken breast, and for bread crumbs and raw pasta (assumption justified by the very similar %TS, %VS and label data for both of the couple of data);
- weight percentage on raw basis for apple-banana mousse (manually varied).

A system of 4 equations and 4 variables (weight percentage on raw basis of tuna/raw chicken breast, butter, lyophilized minestrone soup and bread crumbs/raw pasta) was solved.

Both the four single sub-fractions and the eight mixtures were used for hydrogen production test. All the samples were shredded using a kitchen blender to homogenize and reduce in smaller sizes. Substances were finally stored in refrigerator at 4°C or freezer at -20°C.

All samples were characterized for the following parameters: Total Solid (TS), Volatile Solid (VS), Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), Chemical Oxygen Demand (COD). Samples were also analyzed for the following parameters: lipids, proteins, carbohydrates,

hemicelluloses, cellulose, lignin, non structural carbohydrates (NSC), starch, free sugars, sucrose and glucose.

Table 2. Food products tested.

Sub-fractions	Food products
Meat, Fish and Cheese	Raw chicken breast
	Tuna
	Butter
Fruit	Apple-Banana mousse
Vegetable	Lyophilized minestrone soup
Bread and Pasta	Bread crumbs
	Raw pasta

Table 3. 8-mixtures composition in terms of carbohydrates, proteins and lipids.

	%		
	Carbohydrates	**Proteins**	**Lipids**
MIX 1	65	15	20
MIX 2	55	15	30
MIX 3	45	15	40
MIX 4	35	15	50
MIX 5	65	20	15
MIX 6	55	30	15
MIX 7	45	40	15
MIX 8	35	50	15

Table 4. Data on carbohydrate (Carb), protein (Prot) and lipid (Lip) content of food products. Data from the labels of the products from the producers.

Food product	g/100g_edible part		
	Carb	**Prot**	**Lip**
Tuna	0,00	28,67	0,92
Butter	1,09	0,79	82,68
Raw chicken breast	0,00	24,08	0,83
Apple-Banana mousse	13,35	0,51	0,21
Lyophilized minestrone soup	62,60	15,65	4,21
Bread crumbs	74,26	13,34	4,30
Raw pasta	74,21	13,59	1,57

Table 5. 8-mixtures composition: % of different food products (raw basis).

	MIX							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Meat-Fish-Cheese								
Tuna	3,5	4,9	6,1	7,5	6,7	15,0	23,1	31,1
Butter	7,9	12,7	17,5	22,3	5,5	5,5	5,5	5,5
Raw chicken breast	3,5	4,9	6,1	7,5	6,7	15,0	23,1	31,1
Fruit								
Apple-Banana mousse	61,1	58,7	56,4	54,0	55,7	42,3	29,0	15,7
Vegetable								
Lyophilized minestrone soup	11,4	8,3	8,7	5,6	14,7	10,8	10,5	10,2
Pasta-Bread								
Bread crumbs	6,3	5,2	2,6	1,6	5,4	5,7	4,5	3,2
Raw pasta	6,3	5,2	2,6	1,6	5,4	5,7	4,5	3,2

2.2 Inoculum

Biological hydrogen potential production test were done using two different types of sludge. One was an anaerobic sludge coming from the anaerobic digester of Cà Nordio Waste Water Treatment Plant located in Padova, Italy. The other type was a granular sludge, collected from a full-scale Upflow Anaerobic Sludge Blanket (UASB) anaerobic digester of a brewery factory situated in Padova.

Both sludge were heat-treated in order to select only hydrogen producing bacteria and inhibit hydrogenotrophic methanogens. Different treatment conditions of temperature and residence time were used. Anaerobic sludge was treated at 80°C for 15 minutes on a heating plate magnetic stirrer. Anaerobic granular sludge was heat-treated at 100°C for 4 hours in an oven (Alibardi *et al.*, 2012).

Moreover, sludge were characterized for the following parameters: TS, VS, TKN. Results are reported in Table 6.

Table 6. Sludge characteristics.

	TS	**VS (% of TS)**	**TKN (gN/kgVS)**
Anaerobic sludge	9 (gTS/l)	46	107,6
Granular sludge	10 (% of raw)	82	105,7

2.3 Biochemical Hydrogen Potential (BHP) test in batch reactor

BHP-tests were performed in batch reactors under mesophilic conditions. In these experiments batches were 1l Pyrex vessels, hermetically closed through a plug with a silicon septum that allowed gas and liquid sampling by a syringe. The working volume of the bottle was 500 ml and consisted of substrate, inoculum, phosphate buffer solution and concentrated HCl to set initial pH at a value of 5.5, macro and micro-nutrients and distilled water to reach working volume. Working conditions chosen for BHP-tests are presented in Table 7. Anaerobic conditions were obtained through a 3 minutes flushing of N_2 gas in the head space of bottles. Bottles were incubated without stirring in a thermostatic water bath at steady temperature of 35°C ± 1°C. Blank tests, prepared in the same way described before taking out substrate, were performed in order to measure the sole microorganisms gas production. Each test was carried out in triplicate, while blanks in duplicate. Tests lasted till the end of gas production, this means a duration of about 3 days in the case of anaerobic sludge and a longer one of about 7 days for granular sludge. During this period the quantity and quality of biogas were measured and pH monitored once/twice a day through a litmus paper. At the end of fermentation tests pH was measured by a pH-meter and liquid samples were collected, filtered at 0.2 μm and stored in refrigerator at 4°C. Liquid sample were analyzed for the following parameters: Dissolved Organic Carbon (DOC), ammonium (NH_4^+) and Volatile Fatty Acids (VFAs) concentrations.

The quantity of biogas produced in fermentative process was measured through dislocation method. The biogas produced led to a pressure increase in the head space in batch reactors and, according to the functional principle of dislocation, moved a volume of liquid, present in another connected bottle, equal to the volume of gas produced. The displaced liquid was an acid saline solution (pH < 3 and 25% NaCl), where CO_2 and CH_4 can not dissolve, and was collected in a graduated cylinder to measure the volume quantity. Biogas quality was analyzed through a gas chromatograph.

The volume of hydrogen produced during two consecutive measurements, *t-1* and *t*, was calculated with the following formula:

$$V_{C,t} = C_{C,t} \cdot V_{G,t} + V_H \cdot (C_{C,t} - C_{C,t-1})$$

where:

$V_{C,t}$ is the volume of hydrogen produced in the time interval between *t-1* and *t*;

$C_{C,t}$ and $C_{C,t-1}$ are the hydrogen concentrations measured at *t* and *t-1*, respectively;

$V_{G,t}$ is the volume of biogas produced in the time interval between *t-1* and *t*;
V_H is the volume of reactor headspace.

Data of hydrogen yield (ml/gVS) are expressed as Nml of hydrogen at temperature of 0°C and pressure of 1 atm.

Table 7. Working conditions for BHP-tests.

Working conditions	
Substrate concentration	5 gVS/l
F/M (Food over Microorganisms ratio)	3 gVS/gVS (anaerobic sludge)
	1 gVS/gVS (granular sludge)
Working volume	500 ml
T	35°C ± 1 °C
Initial pH	5.5
Residence time	3-7 days

2.4 BHP test in batch stirred reactor

BHP tests were also performed in batch stirred reactors. The glass bottle used for the experiment had a total volume of 560 ml and a working volume of 450 ml. A heating plate magnetic stirrer was used to continuously mix the reactor (at 250 rpm) and to keep the temperature at a constant value of 35°C (mesophilic condition). The bottle had two exits: one was used to take liquid and gas sample through a silicon plug by suing syringes; the second exit was connected through a plastic pipe to a wet-tip biogas meter. An insulating jacket was provided to limit heat dispersion. Working conditions concerning substrate concentration and F/M ratio were the same already reported in paragraph 2.3 and in Table 7 (5 gVS/l and 3, respectively). Experiments tested two different values of initial pH, 5.5 and 7.0, obtained adding some drops of concentrated HCl or sodium hydroxide to the mixture. Moreover, a specified phosphate buffer solution (250 ml) was added to keep pH to the value of 5.5 or 7.0. A webcam was used to register every 10 minutes the number of turning of the wet-tip gas meter.

Tests were conducted on four selected mixtures (Mix 1, Mix 4, Mix 5, Mix 8) using Cà Nordio anaerobic sludge. Blank tests were also performed.

Liquid samples were taken at the beginning, at the middle and at the end of the experiments, while gas samples only at the middle and at the end of the test. Liquid samples were filtered at 0,2 μm and analyzed for DOC and COD. Gas samples were analyzed through a gas

chromatograph (GC) for hydrogen and carbon dioxide concentration. To calculate the hydrogen production of each test, the quality of gas produced was assumed constant and described by its final concentration in carbon dioxide and hydrogen given by the GC.

Data on biogas production were interpolated using the Gompertz equation (Trzcinski and Stuckey, 2012; De Gioannis *et al.*, 2013):

$$P = P_s \cdot \exp\left(-\exp\left(\frac{R_m \cdot e}{P_s}(\lambda - t) + 1\right)\right)$$

where:

P is the biogas production at time t (Nml);

P_s is the biogas production potential (Nml);

R_m is the maximum biogas production rate (Nml/h);

λ is the duration of the lag phase (h).

Data of biogas production (ml) are expressed as Nml of biogas at temperature of 0°C and pressure of 1 atm.

2.5 Analytical methods

TS, VS, TKN, Nitrogen in the form of NH_4^+ and COD were analyzed according to Standard Methods (APHA, 1999). TOC and DOC was measured using a Total Carbon Analyzer (TOC-V CSN, Shimadzu). VFAs concentrations were measured using a Gas Chromatograph (GC Varian 3900) equipped with a Varian 25m×0.53mm ID CP-WAX 58 column. Nitrogen was used as carrier gas. The biogas composition in the reactor headspace was measured using a micro-GC (Varian 490-GC) equipped with a 10 meter MS5A column and a 10 meter PPU column. Helium was the carrier gas.

3. RESULTS AND DISCUSSION

3.1 Composition and characterization of sub-fractions and food products

Data on the composition of food products used to simulate the organic fraction of municipal solid waste are taken from the product labels. As supposed, food products selected for the sub-fraction 'Meat, Fish and Cheese' are characterized by the high quantity of proteins or lipids (from 97 % to 98 %) while those selected to represent the sub-fractions 'Fruit' and 'Bread and Pasta' are characterized by high quantity of carbohydrates (between 81-95%). The sub-fraction 'Vegetable' contains both carbohydrates and proteins. In Table 8, data on chemical composition of sub-fractions and food products and on total solid, volatile solid and TKN content are reported. All food products are characterized by high content of VS being edible materials and data on TKN content correlate linearly with data on protein content of sub-fractions. Table 9 presents the data on the chemical compound contents of the four sub-fractions.

Table 8. Data on sub-fractions composition (% of raw weight), carbohydrate (Carb), protein (Prot) and lipid (Lip) (% of volatile solids), Total solid (TS), Volatile solid (VS) and Total Kjeldahl Nitrogen (TKN) content of sub-fractions and food products.

Sub-fractions	**Composition (%)**	**Carb (%)**	**Prot (%)**	**Lip (%)**	**TS (%)**	**VS (%)**	**TKN (gN/kgVS)**
Meat, Fish and Cheese		**0**	**55**	**45**	**40**	**97**	**85,7**
Tuna	40	0	97	3	31	95	-
Butter	20	1	1	98	85	100	-
Raw chicken breast	40	0	97	3	26	95	-
Fruit		**95**	**4**	**1**	**16**	**89**	**4,7**
Apple-Banana mousse	100						
Vegetable		**76**	**19**	**5**	**95**	**87**	**20,8**
Lyophilized minestrone soup	100						
Bread and Pasta		**82**	**15**	**3**	**93**	**98**	**22,2**
Bread crumbs	50	81	15	4	94	97	-
Raw pasta	50	83	15	2	90	99	-

Table 9. Chemical composition of the four sub-fractions. All data are reported as percentage of Total Solids (% of TS). All data are characterized by a variability of 5% due to analytical errors.

Chemical compounds	Sub-fractions			
	Meat, Fish and Cheese	Fruit	Vegetable	Bread and Pasta
Lipids	4	0	4	33
Proteins	13	3	11	52
Carbohydrates	82	92	72	12
Hemicelluloses	2	2	3	12
Cellulose	< 1	3	3	< 1
Lignin	< 1	1	< 1	6
NSC*	81	86	66	12
Starch	75	< 1	48	< 1
Free sugars	6	86	18	12
Sucrose	5	< 1	< 1	< 1
Glucose	< 1	44	5	< 1

* Non Structural Carbohydrates (NSC)

3.2 Composition and characterization of 8-mixtures

Eight different mixtures of the four sub-fractions reported in paragraph 3.1 were created. Any mixture was characterized by different percentages of carbohydrates, proteins and lipids as previously reported in Paragraph 2.1. Table 10 presents the data of the physical and chemical characterization of the mixtures for the following parameters: TS, VS, TKN and TOC. Table 11 presents the data on the chemical compound contents of the eight mixtures.

The data on TOC and TKN confirm the chemical composition of the mixtures. The TOC increases from Mix 1 to Mix 4 and this agrees with the growing content of lipids in the mixtures. Similarly the TKN increases from Mix 5 to Mix 8, being the four mixtures characterized by increasing content of protein while TKN remains almost constant from Mix 1 to Mix 4, having theoretical equal content of protein.

The characterization of the chemical compound composition of the eight mixtures reported in Table 11 confirmed the assumptions on the theatrical composition of the mixtures calculated from the specific characteristics of the food products. The analysis confirmed the constant content of protein from Mix 1 to Mix 4 and the constant content of lipids for Mix 5 to Mix 8 and also confirmed the range of variability of the three groups (lipids, protein and carbohydrates) for each of the eight mixtures. The analyses indicate also the low content of hemicelluloses,

cellulose and lignin in the substrates. This is due to the fact that edible food products were used to simulate the OFMSW. The only sub-fraction contributing to the content of hemicelluloses and lignin is "Meat, Fish and Cheese" (Table 11). For all the mixtures the larger proportion of carbohydrates is composed by non structural carbohydrates (di and mono saccaridies). The two mixtures characterized by the highest content of starch are Mix 1 and Mix 4, both composed by large quantities of the sub-fractions "Bread and Pasta" and "Vegetables". The variation of free sugars are more influenced by the sub-fractions "Fruits" which is on the contrary characterized by very low content of starch and large content of free sugars. Glucose represents in all mixtures the main monosaccarides in the free sugar.

Table 10. Physical and chemical characterization of the eight mixtures.

Mixtures	**TS (%)**	**VS (%)**	**TOC (%C on TS)**	**TKN (gN/kgVS)**
1	41	95	42,1	20,1
2	39	96	46,2	21,6
3	40	96	50,9	22,3
4	40	97	54,3	21,8
5	40	94	40,4	27,1
6	39	95	43,0	46,2
7	39	94	45,3	62,5
8	41	95	44,0	75,8

Table 11. Chemical composition of the eight mixtures. All data are reported as percentage of Total Solids (% of TS). All data are characterized by a variability of 5% due to analytical errors.

Chemical compounds	Mixtures							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Lipids	15	26	39	48	15	15	15	15
Proteins	12	13	13	13	16	27	37	45
Carbohydrates	68	57	44	36	63	53	43	34
Hemicelluloses	6	8	7	6	5	5	5	6
Cellulose	2	2	2	3	2	1	1	1
Lignin	3	6	4	3	2	2	3	2
NSC*	58	47	23	23	55	45	35	26
Starch	28	21	12	5	26	22	17	11
Free sugars	30	26	19	18	29	23	18	15
Sucrose	7	6	5	3	8	8	7	5
Glucose	15	14	14	12	15	13	9	6

* Non Structural Carbohydrates (NSC)

3.3 BHP test on single categories and on 8-mixtures in batch reactor

Table 12 and Table 13 show the results obtained from BHP tests on single categories and on the eight mixtures obtained utilizing anaerobic and granular sludge respectively. The values obtained utilizing granular sludge are lower than those obtained with anaerobic sludge. This difference is particularly higher for the eight mixtures. Moreover, granular sludge shows a slower kinetic: while gas production in tests with anaerobic sludge ended in 2 days, granular sludge took between 3 and 6 days to finish the fermentation. This can be explained by the fact that the anaerobic sludge is a flocculent type biomass. Therefore the distribution of inoculum in the reactor is more homogenous allowing a higher contact between bacteria and substrate. Granular sludge on the contrary is characterized by fast settleability and bacteria are grouped in complete communities only in the granule. The contact between the substrate and inoculum is more limited and distribution of organics to be degrades is mainly guided by diffusion effects without constant mixing of the reactors. This effect influenced therefore both the hydrolysis and the hydrogen production rates characterizing the lower and slower hydrogen production from tests with granular sludge.

Nevertheless, the effect of the mixture composition on hydrogen production are similar for both inoculum. Biogas and hydrogen productions in fact resulted linearly correlated to

carbohydrates content for the four single sub-fractions and for the eight mixtures as shown in Figure 1 and Figure 2. The percentage of hydrogen in the biogas resulted in the range of 43 % to 57%, except for the sub-fraction 'Meat, Fish and Cheese' where it resulted from 3 % to 4%. In addition, it is possible to notice that the mixtures 5 to 8 have generally higher biogas and hydrogen yields than those obtained from Mix 1 to Mix 4, together with a slightly higher % of H_2 in the biogas. This could be explained by a positive effect of protein contents on hydrogen producing metabolic pathways if compared to the presence of lipids.

Final pH values show a correlation with gas production: pH decreases with increasing gas production, due to the formation of VFAs during the fermentation. Additionally, the higher value of final pH corresponds to the substrate with higher content of proteins ('Meat, Fish and Cheese' and Mix 5-8) as these chemical compounds yield good buffering capacity due to the production of ammonia (Elbeshbishy and Nakhla, 2012).

Data of hydrogen yields obtained from single sub-fractions are in accordance with results obtained by Dong *et al.* (2011) and Okamoto *et al.* (2000). Both authors made experiments on single food products (see Table 1) that simulate the organic fraction of municipal solid waste. Dong *et al.* (2011) reported a value of 125 mlH_2/gVS for rice and 0 mlH_2/gVS for lean meat. Similar data were obtained in this study from the sub-fractions "Bread and Pasta" and "Meat, Fish, Cheese". Mix 1 and Mix 5 could be considered the most comparable mixtures to a general OFMSW in terms of product content and chemical composition. Boni *et al.* (2013) found a value of 70,34 mlH_2/gVS for food waste and it is in accordance with results found in this study, considering that substrates tested were fresh food, not waste, that could present a greater potential.

Finally, an estimation of hydrogen production of the eight mixtures was made using the results of hydrogen yields of single categories. Knowing the exact composition of each mix and sub-fraction in terms of food products, the yields of single sub-fractions were multiplied for the grams of VS of that fraction present in each mixture. The calculated value was compared with the experimentally measured yields of each mixture. From Figure 3 it can be observed that the error between the two values resulted very small and data stay on the line 45° line ($y=x$). It is interesting to highlight that real values are always higher than the estimated ones, except for Mix 1. Data in the graph are in fact over the 45° line. Moreover, Mix 5-8 compared to Mix 1-4 show a greater increase of real values on calculated ones, 4-10% against -1-6%. This, again, confirms the positive role of proteins in biological hydrogen fermentation. For BHP tests using granular sludge this is not confirmed and data have a high variability as shown in Figure 3 (right side).

Table 12. Biogas and hydrogen potential production and final pH values of BHP tests utilizing anaerobic sludge.

Substrate	Biogas yield (Nml/gVS)	H2 yield (Nml/gVS)	% H2	Final pH
Meat, Fish and Cheese	29 ± 2	0,7 ± 0,6	3%	5,69 ± 0,03
Fruit	359 ± 3	189 ± 2	53%	4,65 ± 0,01
Vegetable	279 ± 6	150 ± 1	54%	4,81 ± 0,01
Bread and Pasta	308 ± 2	168 ± 2	54%	4,61 ± 0,02
MIX 1	245 ± 3	129 ± 0,3	53%	4,50 ± 0,03
MIX 2	212 ± 3	113 ± 2	53%	4,53 ± 0,07
MIX 3	177 ± 1	94 ± 0,3	53%	4,68 ± 0,04
MIX 4	129 ± 0,6	70 ± 0,5	54%	4,80 ± 0,03
MIX 5	258 ± 5	136 ± 2	53%	4,58 ± 0,04
MIX 6	218 ± 1	120 ± 4	55%	4,67 ± 0,15
MIX 7	171 ± 8	93 ± 3	54%	4,82 ± 0,02
MIX 8	137 ± 4	77 ± 0,2	56%	5,03 ± 0,03

Table 13. Biogas and hydrogen potential production and final pH values of BHP tests utilizing granular sludge.

Substrate	Biogas yield (Nml/gVS)	H2 yield (Nml/gVS)	% H2	Final pH
Meat, Fish and Cheese	25 ± 3	0,9 ± 0,8	4%	5,72 ± 0,01
Fruit	350 ± 32	168 ± 11	48%	4,33 ± 0,02
Vegetable	254 ± 5	124 ± 5	49%	4,52 ± 0,06
Bread and Pasta	227 ± 17	117 ± 6	51%	4,28 ± 0,04
MIX 1	130 ± 8	57 ± 4	44%	4,67 ± 0,06
MIX 2	104 ± 3	44 ± 3	43%	4,67 ± 0,01
MIX 3	97 ± 7	49 ± 5	51%	4,80 ± 0,04
MIX 4	80 ± 5	41 ± 2	52%	4,85 ± 0,04
MIX 5	125 ± 32	66 ± 15	52%	4,56 ± 0,09
MIX 6	168 ± 11	94 ± 8	56%	4,62 ± 0,03
MIX 7	135 ± 23	76 ± 14	57%	4,77 ± 0,05
MIX 8	103 ± 1	59 ± 1	57%	4,89 ± 0,03

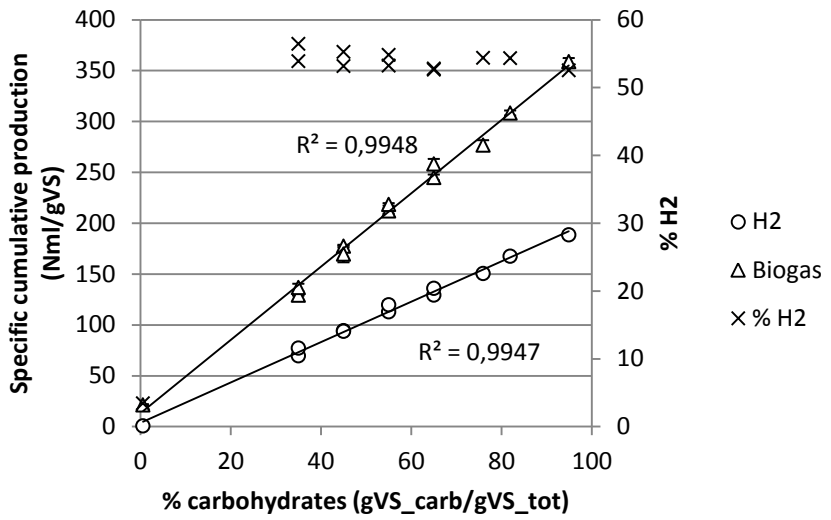

Figure 1. Specific cumulative gas production (Nml/gVS) correlated to carbohydrates content in BHP tests on single categories and 8-mixes utilizing anaerobic sludge.

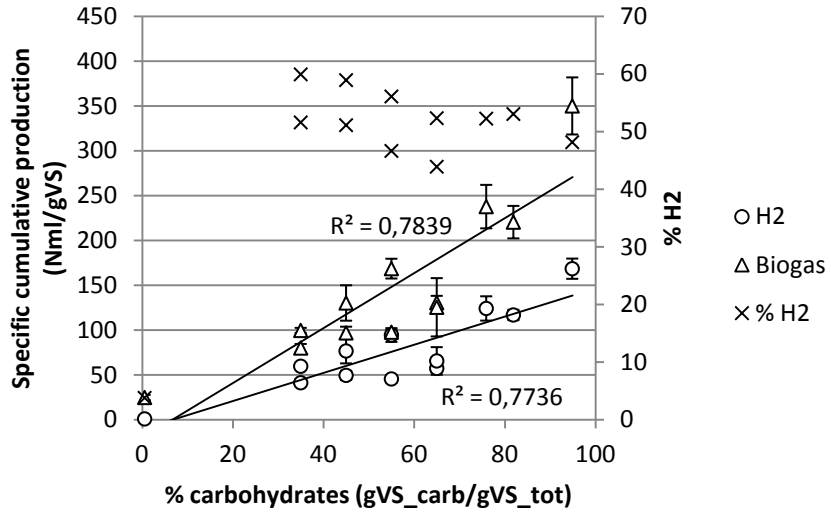

Figure 2. Specific cumulative gas production (Nml/gVS) correlated to carbohydrates content in BHP tests on single categories and 8-mixes utilizing granular sludge.

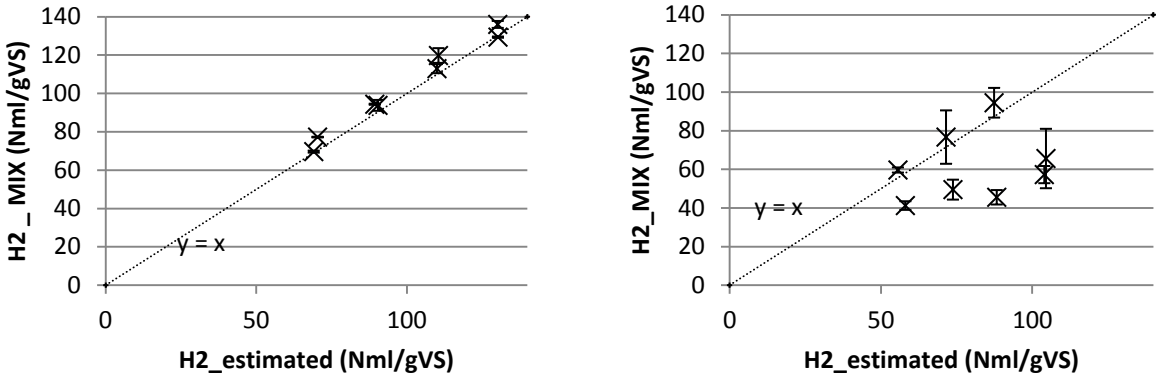

Figure 3. Comparison between the volume of hydrogen produced by the 8-mixtures and the sum of the hydrogen volumes produced by their single fractions using anaerobic sludge (left side) and granular sludge (right side).

At the end of BHP tests, liquid samples were taken and filtered at 0,2 μm and DOC, Nitrogen and VFAs concentrations were analyzed. Data are reported in Annex 2 for anaerobic sludge and granular sludge tests. DOC concentrations are quiet similar using both types of inocula, except for Mix 5-8 where values are a bit higher utilizing granular sludge. DOC correlates well with carbohydrates content. DOC concentration increases when the % of carbohydrates increases as shown in Figure 4. The only exception is sub-fraction 'Meat, Fish and Cheese' that is characterized by 1% of carbohydrates but a percentage of lipids of 45% that could explain the quite high value of DOC.

Data about Nitrogen concentrations are also similar between tests with the two different sludge as regards to single sub-fractions, while for what concerns the eight mixtures tests values obtained utilizing granular sludge are a slightly higher than those obtained from anaerobic sludge. Taking into consideration the single sub-fractions and the 8 mixtures, a linear correlation between final Nitrogen concentrations and initial % of proteins can be observed and related to the hydrolysis of proteins. Similar results were reported by Elbeshbishy and Nakhla (2012). This is depicted in Figure 5. Also blank tests correlate linearly: the assumption of biomass formula $C_5H_7O_2N$ (Elbeshbishy and Nakhla, 2012) was made and a percentage of 12,4% of $mol_N/mol_{C5H7O2N}$ was calculated and assumed as '% of proteins'. Furthermore, it is important to consider that part of the hydrolyzed Nitrogen is consumed in bacterial growth as nutrient. Blank tests are in the endogenous phase, so the amount of NH_4^+ used for growth is very low, and this is another explanation for higher values of ammonium than in other tests on single sub-fractions and mixtures. The high nitrogen release measured for the sub-fraction 'Meat, Fish and Cheese',

could be explained by the high protein content (55%) and the low NH_4^+ utilization for bacterial growth being hydrogen fermentation and gas production very limited.

Regarding VFAs concentrations, data are quiet comparable between experiments conducted with anaerobic or granular sludge. Concentrations in blank tests are not significant. In BHP tests on single sub-fractions and 8 mixtures the most relevant data are these of acetic acid and butyric acid concentrations, as reported by Dong *et al.* (2011), Okamoto *et al.* (2000), Boni *et al.* (2013), Bai *et al.* (2004) and Liu *et al.* (2006). Boni *et al.* (2013) analyzed the co-digestion of slaughterhouse waste (SHW) and food waste (FW) in fermentative H_2 production testing nine mixtures with different proportions of FW and SHW. The study showed that acetic and butyric acid were the predominant soluble metabolites, with lower production of propionic acid. Moreover, the measured butyrate mostly exceed acetate (as mg kg^{-1} of digestate).

In the scientific work of Bai *et al.* (2004), multiple substrates containing different ratios of glucose and peptone were utilized to investigate the roles played by carbohydrate and protein in hydrogen fermentation. An accumulation of acetate was observed with increasing peptone content in multiple substrates. Acetate was the main product of the fermentation of peptone, while glucose degradation led to both acetate and butyrate as byproducts.

Liu *et al.* (2006) tested household solid waste (HSW) in the two-stage fermentation process and studied the influence of pH on the metabolic pathways selection by hydrogen producing microorganisms. It was noticed that when pH was at 5.2 highest hydrogen production was found and acetate was the almost only end-product, while when pH dropped to 4.8 less hydrogen was produced and butyrate started to accumulate. Then when pH recovered to 5.2 butyrate dropped and hydrogen production increased.

In this research study, as shown in Figure 6 and Figure 7, concentrations of butyric acid results always higher or equal to those of acetic acid with the exception of 'Meat, Fish and Cheese' category, Mix 4 and Mix 8. These three substrates showed in fact opposite trend being the substrates with lower carbohydrates content and gas production. Moreover, a general trend for acetate and butyrate can be observed. Higher concentrations were measured for Mix 5-8 if compared to Mix 1-4. Increasing concentrations of acids were measured at increasing % of carbohydrates in the substrates, that is in turn related with increasing gas production. Dong *et al.* (2011) and Bai *et al.* (2004) reported that the production of acetate and butyrate was strongly associated with that of hydrogen. Finally, low concentrations of propionate were measured except for the sub-fraction 'Meat, Fish and Cheese' and in Mix 5-8. This fact could be associated to the higher % of proteins in these substrates. Caproic acid shows significant concentration in

Mix 1-5, but only in tests that used granular sludge. Isovaleric acid and isobutyric acid show significant values only in 'Meat, Fish and Cheese' category.

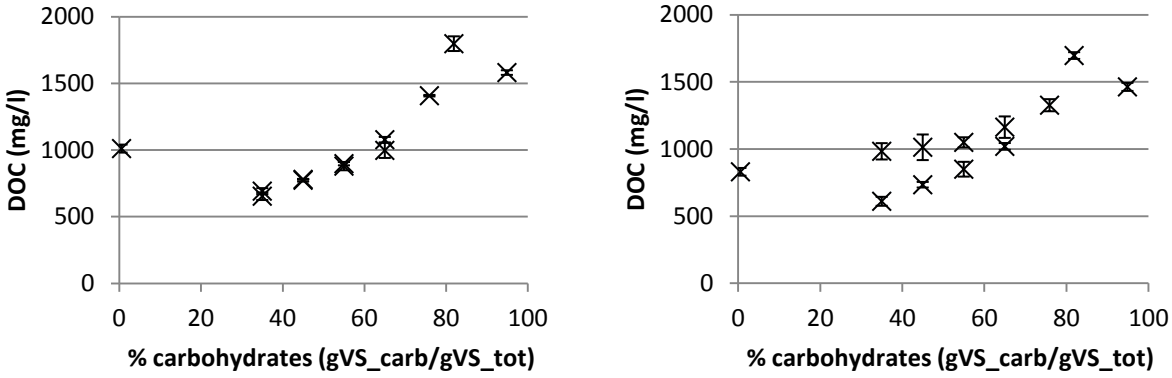

Figure 4. DOC concentrations net of blank values for BHP tests using anaerobic sludge (left side) and granular sludge (right side).

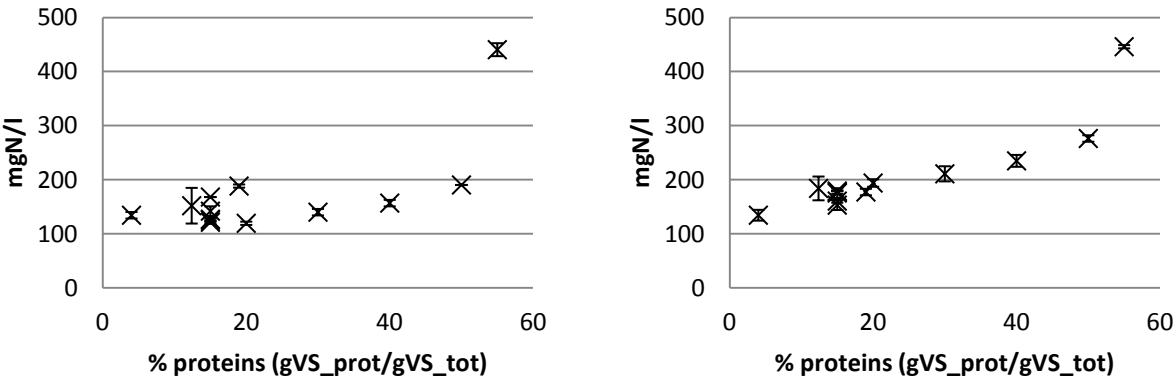

Figure 5. Ammonium concentrations for BHP tests using anaerobic sludge (left side) and granular sludge (right side).

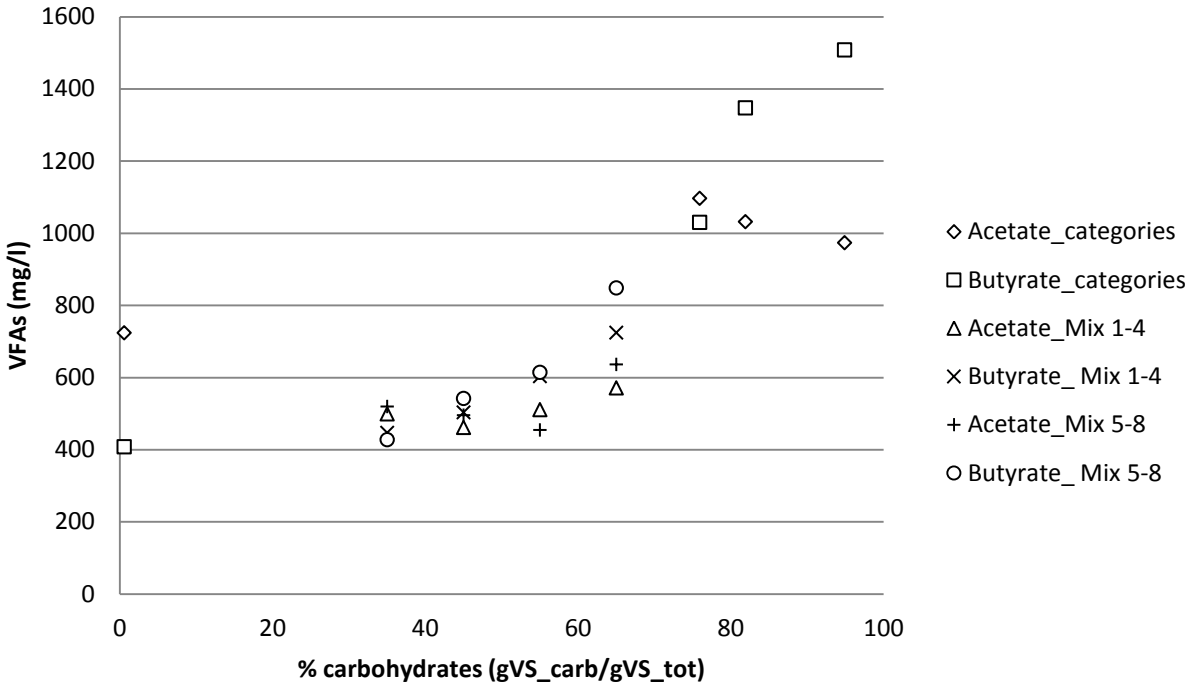

Figure 6. VFAs concentrations net of blank values for BHP tests using anaerobic sludge.

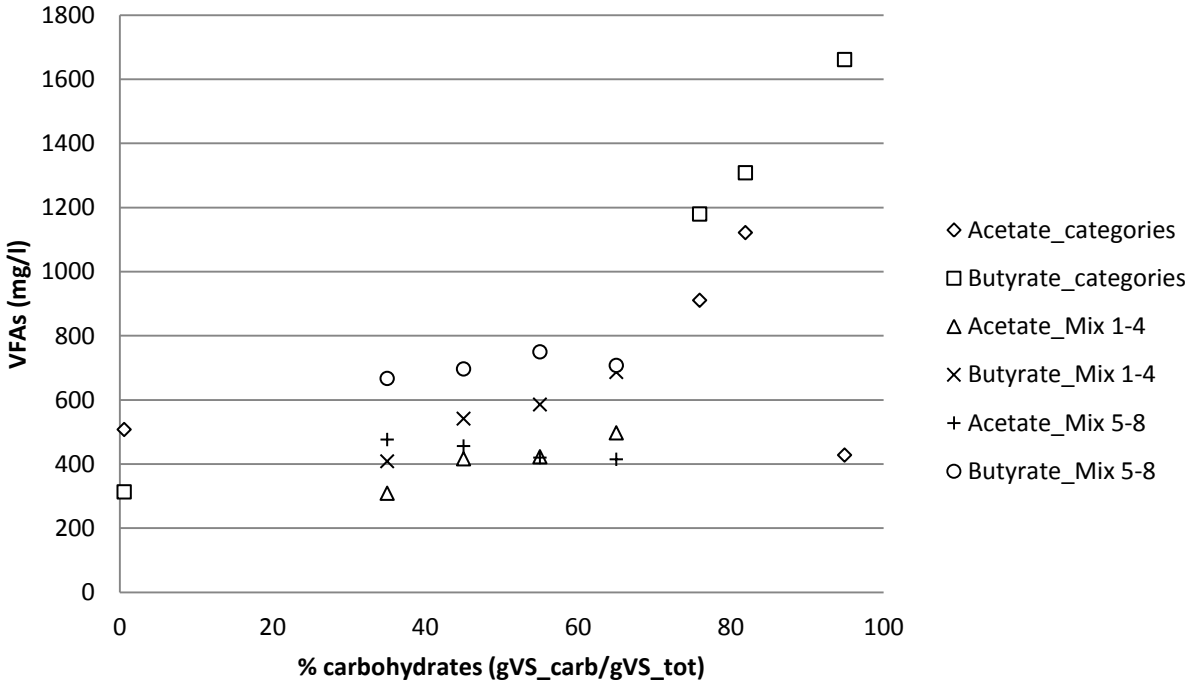

Figure 7. VFAs concentrations net of blank values for BHP tests using granular sludge.

An estimation of the % of degradation of Carbon was done using the data on carbon dioxide production, dissolved organic carbon and inorganic carbon concentrations in the liquid phase of BHP tests. Data are reported in Table 14, Figure 8 and Figure 9. Initial Carbon was calculated through measured data of TOC (gC/gTS) and gTS tested for each mixture. Then the percentages of Carbon hydrolyzed and gasified to CO_2 were calculated; the complementary was residual Carbon not hydrolyzed nor fermented. Results indicate that both the percentages of carbon that is gasified and that is hydrolyzed follow the content of carbohydrates in the substrate. At higher carbohydrate contents corresponds also higher hydrolysis of carbon and consequently higher gasification to carbon dioxide. Comparing the data obtained for mixtures Mix 1-4 and Mix 5-8, results indicate that higher contents of protein lead to higher carbon degradation, confirming the positive effects of proteins on fermentation processes already reported previously.

Table 14. % of degradation of C at the end of BHP tests.

	Anaerobic sludge % C degradation	**Granular sludge % C degradation**
MIX 1	62%	53%
MIX 2	51%	42%
MIX 3	39%	33%
MIX 4	30%	26%
MIX 5	64%	63%
MIX 6	53%	57%
MIX 7	43%	50%
MIX 8	37%	47%

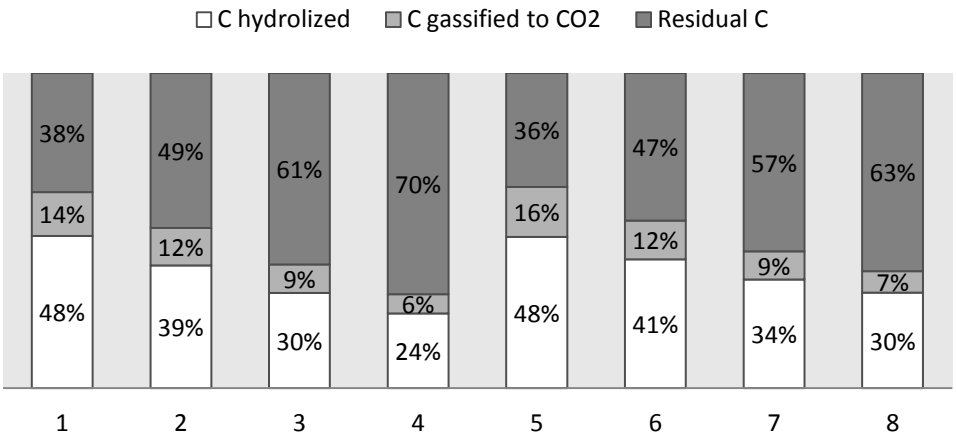

Figure 8. C degradation at the end of BHP tests utilizing anaerobic sludge.

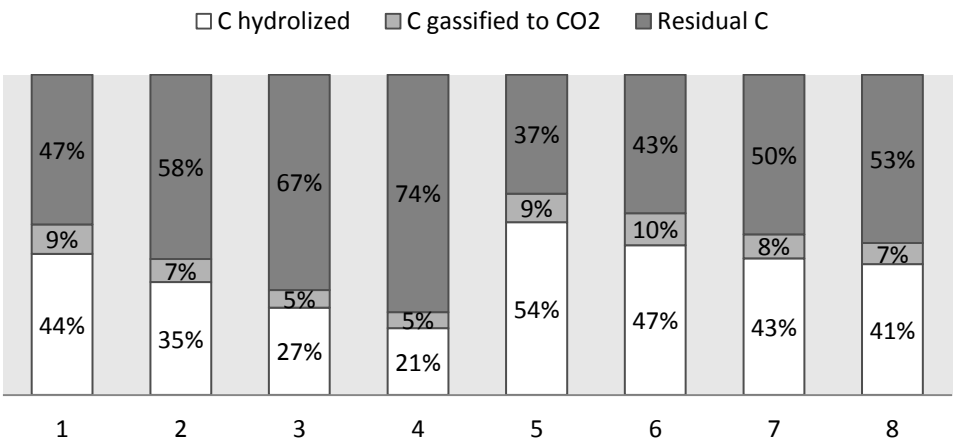

Figure 9. C degradation at the end of BHP tests utilizing granular sludge.

3.4 BHP test on four selected mixtures in batch stirred reactor

In this second part of the research study four mixtures were selected and BHP tests were performed under continuously stirred conditions. Anaerobic sludge coming from Cà Nordio WWTP was chosen between the two different types of sludge, because in previous experiments it showed a better performance compared to the granular sludge in terms of volume of gas produced and velocity of reaction. The tested mixtures were: Mix 1, Mix 4, Mix 5 and Mix 8. They are the mixtures with higher content of carbohydrates (Mix 1 and Mix 5), lipids (Mix 4) and proteins (Mix 8), for the exact percentages of their composition see Table 3. The aim was to better analyze the influences of these chemical compounds in the biological hydrogen fermentation.

Stirred conditions provided continuous contact between substrate and microorganisms, avoided sedimentation and let substances be more available. In this way, the reaction was faster and gas production ended in less than 24 hours while in previous experiences with no-stirred tests it took around 2 days.

Two initial pH values were tested: 5.5 and 7.0. Data of biogas production at pH 5.5 confirmed those obtained in no-stirred experiments at the same conditions. Differently from previous test however, blank tests gave no biogas production. Table 15, Table 16 and Figure 10 show data about biogas production. In Table 15 biogas and hydrogen yields are reported together with their percentages of error relative to values obtained with no-stirred tests at pH 5.5. Moreover the H_2 percentage in the biogas and final pH values are listed.

As already stated, data about biogas and hydrogen production are similar between the two different types of tests, anyway slightly smaller in the continuously stirred ones. By the way, the

percentage of H_2 is higher for Mix 4 and Mix 8 and lower for Mix 1 and Mix 5 compared to no-stirred tests, highlighting better hydrogen fermentation for mixtures with lower carbohydrates contents. However general considerations are the same for both test types: gas production increases with increasing carbohydrates content and Mix 5 and Mix 8 (where proteins content augments) perform better than Mix 1 and Mix 4. Final values of pH are again inversely related to the gas production and slightly lower than the ones obtained in previous experiments. In addition, tests conducted at pH 5.5 clearly obtained better results than the ones at pH 7.0, about double values and even more. Liu *et al.* (2006) analyzed the short-term effect of pH on hydrogen generation testing batch experiments of pH from 3.5 to 8.5 with 0.5 intervals. The highest H_2 production was always at pH 5.5 in the whole experimental period, but after 60 h, pH 5 had a very similar hydrogen value as the one at pH 5.5, indicating the optimum pH should be around 5-5.5. Only for Mix 5 and Mix 8 tests without inoculum addition were performed. Their hydrogen production was zero and biogas yields were very low, probably due to the presence of some bacterial species introduced through substrate in the bottle. The results agrees with previous experiments for hydrogen production from organic waste without the use of an external inoculum (Favaro *et al.*, 2013) where hydrogen production started after a lag-phase of about 5 days by the action of indigenous bacteria. The very low biogas production and the absence of hydrogen fermentation could be related to the very low content of indigenous bacteria from the food products used to simulate the organic waste confirming anyway the long lag phase observed by Favaro *et al.* before hydrogen fermentation could naturally occur.

Final values of pH were much lower than in other tests, describing the good buffering capacity of sludge. Moreover Mix 8 had a final pH of 4.16 higher than the one of Mix 5 of 3.45, confirming the results obtained with no-stirred tests.

Data were interpolated through Gompertz equation as explained in paragraph 2.4 in Chapter 2. The parameters P_s, R_m and λ were estimated by applying a least squares fit of the equation to the experimental data set (Trzcinski and Stuckey, 2012). Data are shown in Table 16 and Figure 10. The duplicates of each test presented a much slower reaction compared to the related first experiments. Indeed the calculated R_m values are generally less than a half of the first test. The greater difference can be observed in Mix 4 and Mix 8 at pH 7.0. An explanation of this trend could be the fact that sludge was shocked every two days, so first experiments were conducted with sludge just heat-treated while duplicate tests utilized sludge shocked one day before. For this reason, data analysis is conducted only on first tests results that are considered more reliable. They are plotted in Figure 10. Data confirm again what was observed in the experiments utilizing no-stirred batch reactors. In particular R_m value, that represents the maximum velocity

of biogas production, is higher for Mix 1 and Mix 5 (high carbohydrates content, 65%) at pH 5.5, 28 and 27 Nml_biogas/(gVS*h) respectively. Moreover, Mix 8 and Mix 4 have values of 24 and 20 Nml_biogas/(gVS*h) respectively at pH 5.5, confirming the better performance of substrate with high proteins content (50%) compared to the one with high lipids content (50%). BHP tests conducted at pH 7.0 show lower R_m values, but always higher for Mix 1 and Mix 5 and in this case the value of Mix 4 is slightly higher compared to Mix 8. Mix 5 and Mix 8 with no inoculum have the lowest values of 3 and 1 Nml_biogas/(gVS*h) respectively. Lag phase duration, λ, has values in the range 5-8 hours and Mix 8, both at pH 5.5 and 7.0, shows to have the lowest values. Calculated values for P_s, the specific biogas production potential, confirm previous observation about gas production.

Furthermore, a method was implemented to determinate the hydrolysis constant, k_h. Trzcinski and Stuckey (2012) individuated two ways to calculate it studying the anaerobic digestion in BMP (Biochemical Methane Potential) test of MSW: they used the first-order model

$$Y = Y_{max} \cdot (1 - \exp(-k_h \cdot t))$$

and assume that methane or soluble COD (SCOD) production followed it. Then the value for k_h was estimated by plotting $ln\left(1 - \frac{Y}{Y_{max}}\right)$ versus time. On the other hand, in this research study, the first-order model was applied to hydrolyzed Carbon, given by the sum of Carbon released in the liquid (DC, dissolved carbon) and gasified to CO_2. The maximum value was assumed to be the TOC measured on solid sample. Unfortunately, it was found out that data collected during BHP tests were not sufficient to this scope. Three samples were taken at t=0, t around 4-7 hours and at the end of experiment. Data of the first two were almost the same because of lag phase duration of hydrogen fermentation. However, it would be important to have frequent sampling during the phase of biogas production to collect a quite high number of data to plot on the graph and obtain a precise line, which angular coefficient is the hydrolysis constant. It is more difficult in hydrogen fermentation in comparison to methane fermentation, due to a huge difference in process duration, about 24 hours and 30 days respectively.

Table 15. Biogas and hydrogen potential production and final pH values of BHP tests in stirred reactor.

	Biogas yield (Nml/gVS)	% error	H2 yield (Nml/gVS)	% error	% H2	Final pH
M 1 - pH 5.5	214	-13%	104	-20%	48	4,36
M 1 - pH 7.0	125		65		52	5,97
M 4 - pH 5.5	106 ± 1	-18%	65 ± 0,2	-7%	61	4,76 ± 0,01
M 4 - pH 7.0	24 ± 13		10 ± 15		42	6,40 ± 0
M 5 - pH 5.5	247 ± 27	-4%	122 ± 9	-10%	49	4,37 ± 0,08
M 5 - pH 7.0	131 ± 5		66 ± 1		50	6,02 ± 0,01
M 5 - no inoculum	18		0		0	3,45
M 8 - pH 5.5	125 ± 2	-8%	75 ± 0	-3%	60	4,73 ± 0,12
M 8 - pH 7.0	46 ± 15		28 ± 9		61	6,43 ± 0,05
M 8 – no inoculum	15		0		0	4,16

Table 16. Three parameters of the Gompertz equation applied to data of biogas production.

	Ps (Nml_biogas/gVS)	Rm (Nml_biogas/(gVS*h))	λ (h)
M 1 - pH 5.5	212	28	7
M 1 - pH 7.0	126	20	6
M 4.1 - pH 5.5	103	20	7
M 4.2 - pH 5.5	122	10	9
M 4.1 - pH 7.0	33	14	8
M 4.2 - pH 7.0	14	3	6
M 5.1 - pH 5.5	230	27	6
M 5.2 - pH 5.5	299	20	10
M 5.1 - pH 7.0	129	21	6
M 5.2 - pH 7.0	147	17	8
M 5 - no inoculum	19	3	8
M 8.1 - pH 5.5	121	24	6
M 8.2 - pH 5.5	143	9	6
M 8.1 - pH 7.0	56	12	5
M 8.2 - pH 7.0	39	2	6
M 8 - no inoculum	14	1	5

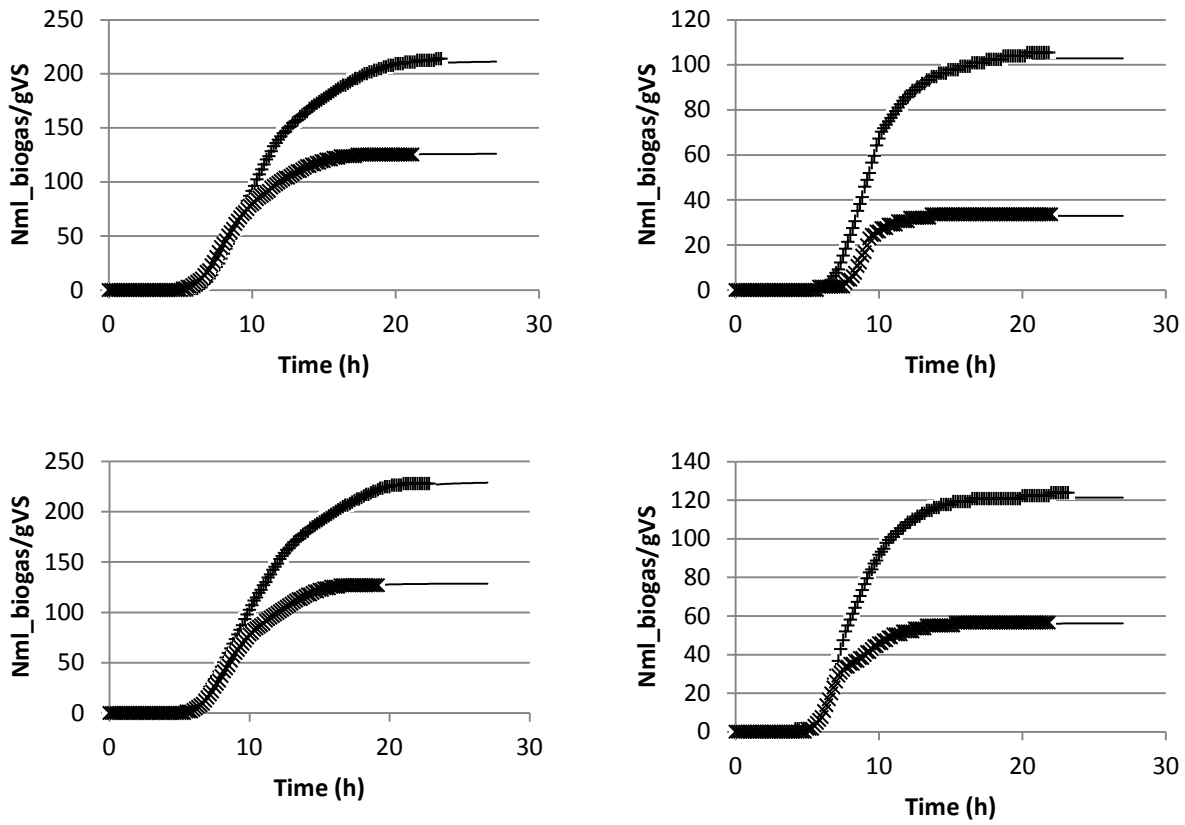

Figure 10. Experimental data at pH 5.5 (+, upper curve) and pH 7.0 (×, lower curve) and calculated data through Gompertz equation (continuous line) for Mix 1 (upper left), Mix 4 (upper right), Mix 5 (lower left) and Mix 8 (lower right).

As regards liquid samples, DOC and COD concentrations were analyzed. TOC and COD were measured also on solid samples and final percentages of degradation were estimated. Taking into account COD, a mass balance was determined calculating the percentage of COD dissolved into the liquid, the percentage of COD gasified to H_2 and the amount of residual COD for difference. The same was done for Carbon degradation, considering the quantity of C in produced CO_2 for the gasified fraction. Results are shown in Figure **11** and Figure **12**. It is possible to notice that, both for COD and Carbon, the gasified fraction decreases at pH 7.0 compared to pH 5.5 for every mixture, while dissolved fraction follows the opposite trend, it increases at pH 7.0 compared to pH 5.5. The first observation is in accordance with data about gas production that is higher at pH 5.5 in respect to experiments at pH 7.0. Non-inoculated tests gave zero hydrogen yields. On the other hand, the augment of dissolved fraction at pH 7.0 could be explained by the decrease in biological activity (lower gas production) and the consequent smaller consumption of organic substance that remains in solution. Data about percentage of Carbon degradation generally confirm results obtained in no-stirred tests.

In general Mix 1 and Mix 5 show the highest percentages of degradation and Mix 8 has a slightly higher one compared to Mix 4. This confirms results previously obtained, that is that substrates rich in carbohydrates show the best performance in biological hydrogen fermentation and that proteins rich substrates performs better than lipids rich ones.

pH and substrate chemical composition greatly affect the process of biological hydrogen fermentation. Lay and Fan (2003) reported that pH value of 6.0 could be indicated for the conversion of fats and proteins rich high-solid organic wastes (HSOW) to H2, while pH 5.0 could be the optimal value for carbohydrates rich HSOW degradation.

Dong *et al.* (2011) listed the different values of hydrolysis constants of carbohydrates, proteins and lipids: 0.025-0.200, 0.015-0.075 and 0.005-0.010 d^{-1} respectively.

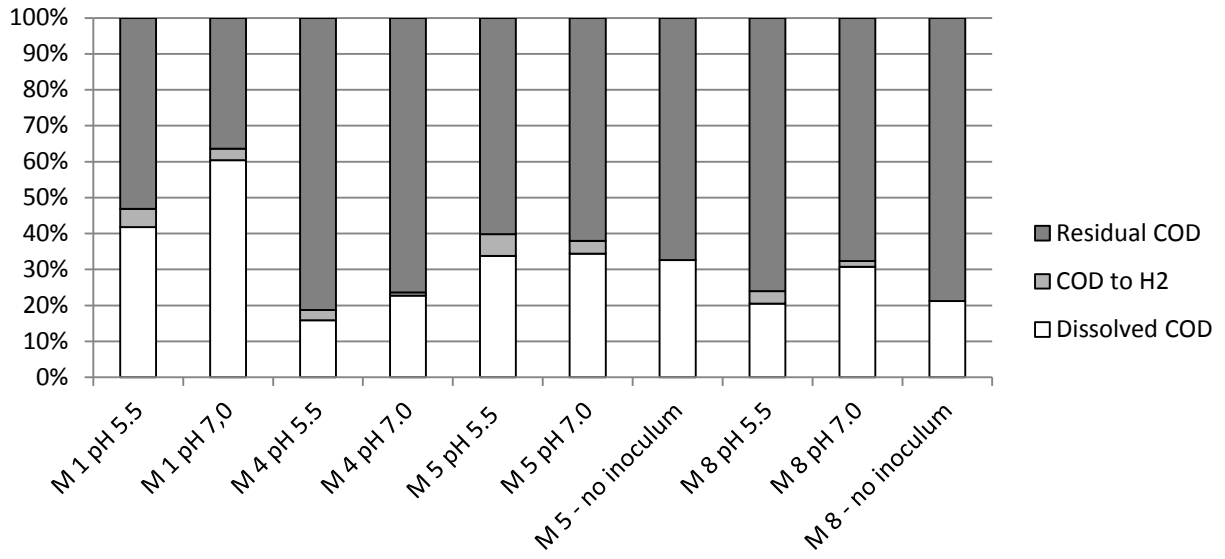

Figure 11. COD balance at the end of BHP continuously stirred tests.

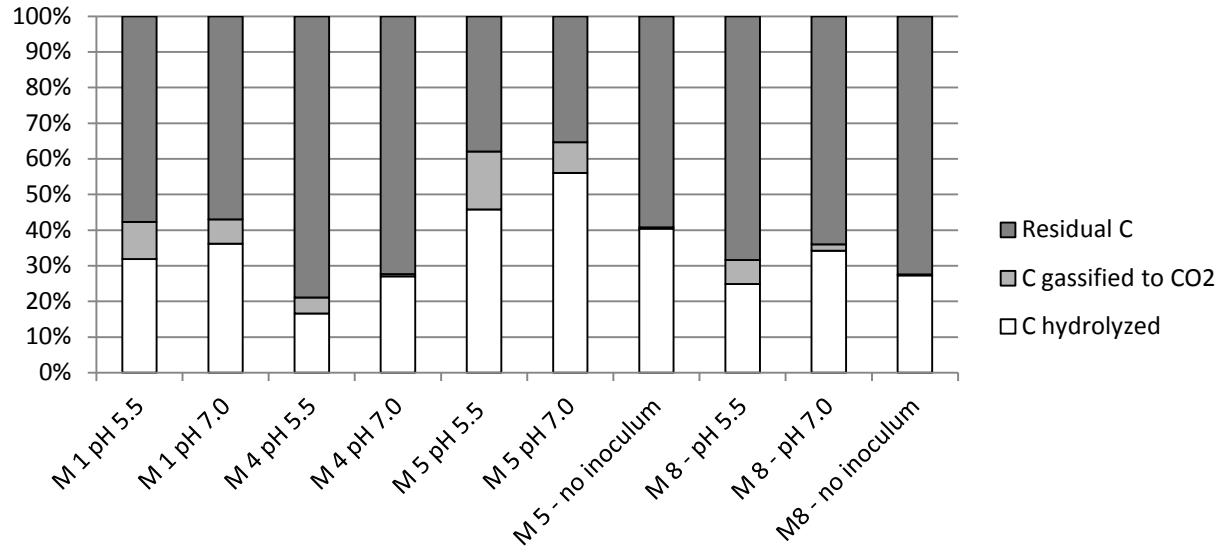

Figure 12. C degradation at the end of BHP continuously stirred tests.

4. CONCLUSIONS

This scientific research studied the biological hydrogen fermentation process of OFMSW using fresh food products as substrate to simulate OFMSW. The aim was to have a perfectly reproducible substance for experiments. Specific products were selected to represent the sub-fractions of OFMSW and 8-different mixtures were defined to analyze the influence of chemical composition of substrate on hydrogen production, considering carbohydrates, proteins and lipids content.

The experimental work was divided in two parts. Firstly BHP tests were performed on the sub-fractions of OFMSW and on the 8-different mixtures in batch reactors using two different types of sludge, anaerobic sludge and granular sludge. Mesophilic conditions were provided and pH was kept constant to the value of 5.5. Secondly four mixtures were selected and tested in continuously stirred batch reactors using anaerobic sludge. Mesophilic conditions were provided and two values of pH were tested, 5.5 and 7.0.

The main results were the following:

- Data about biogas and hydrogen yields at pH 5.5 were the same using no-stirred batch reactors or continuously stirred batch reactors;
- Anaerobic sludge showed to perform better than granular sludge in terms of volume of gas produced and velocity of reaction;
- Biogas and hydrogen yields presented a direct linear correlation with the content of carbohydrates in the substrate. Moreover, Mix 5-8, that had higher proteins content than Mix 1-4, had greater hydrogen yields and better biogas quality. Tests conducted at pH 7.0 obtained about half values of biogas production. Zero hydrogen and very low biogas generation was detected for non-inoculated tests;
- It was possible to estimate the hydrogen production of 8-mixtures considering their composition in terms of sub-fractions and knowing the H_2 yields of the latter. Mix 5-8, compared to Mix 1-4, presented a greater increase of experimental values on calculated ones;
- DOC correlated linearly with carbohydrates content but also lipids had an important role on the results of this analysis;
- NH_4^+ concentrations resulted higher when the proteins content in the substrate was higher;
- Regarding VFAs concentrations, acetic acid and butyric acid were the most abundant and generally butyrate showed higher or equal values than acetate. Moreover, higher values of acetate and butyrate were detected for Mix 5-8 compared to Mix 1-4 and concentrations of these acids increased with increasing percentage of carbohydrates;

- The percentages of carbon and COD degradation, hydrolyzed and gasified, showed to be higher in substrate with higher carbohydrates content. Moreover, Mix 5-8 showed a slightly better performance than Mix 1-4.

In conclusion, a direct linear correlation was found between biogas and hydrogen production and carbohydrates content in the substrate. Moreover, a positive effect of proteins content was observed on hydrogen fermentation if compared to the presence of lipids. Finally, both types of experiments, no-stirred batch and continuously stirred batch, led to same results and could be used to study the biological hydrogen fermentation process.

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ANNEXES

Annex 1

Cumulative hydrogen production of sub-fractions (BP: Bread and Pasta; FR: Fruit; VEG: Vegetable; MFC: Meat, Fish and Cheese) and of 8-mixtures from average experimental data (symbols). Error bars represent the standard deviation of experimental data.

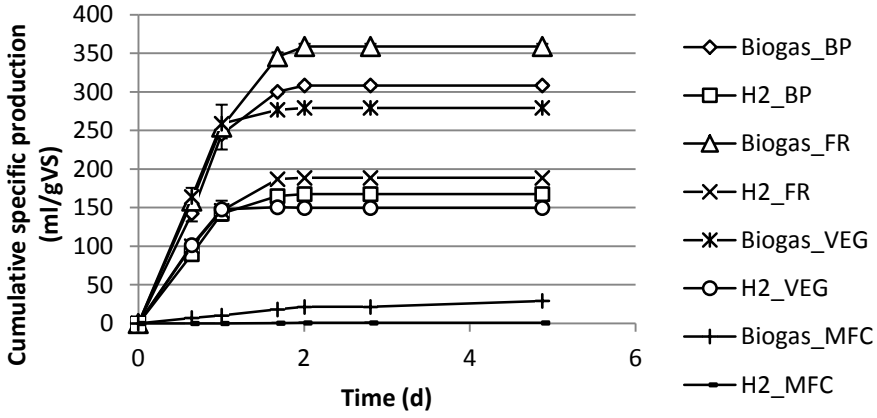

Figure 1.1 Biogas and H_2 production of sub-fractions using anaerobic sludge.

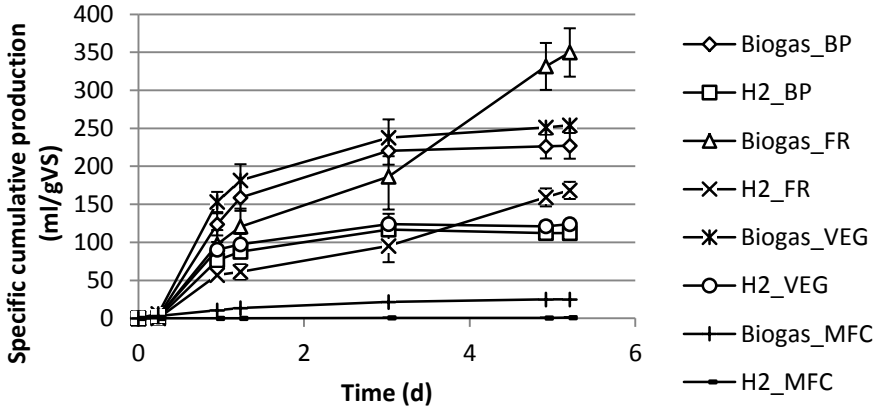

Figure 1.2 Biogas and H_2 production of sub-fractions using granular sludge.

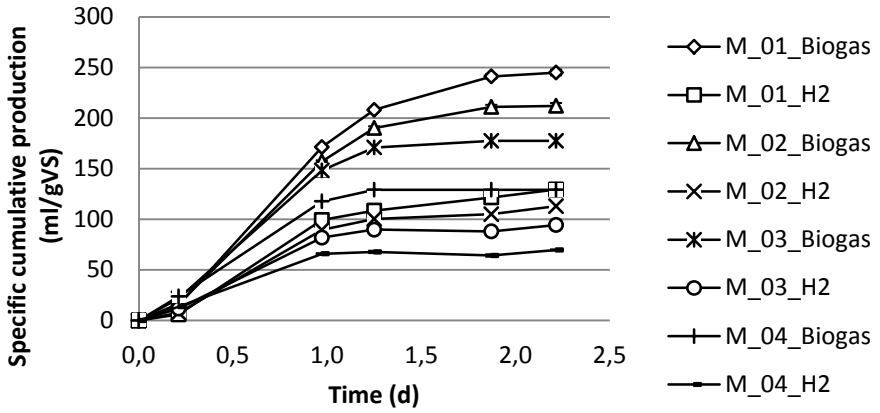

Figure 1.3 Biogas and H_2 production of Mix 1-4 using anaerobic sludge.

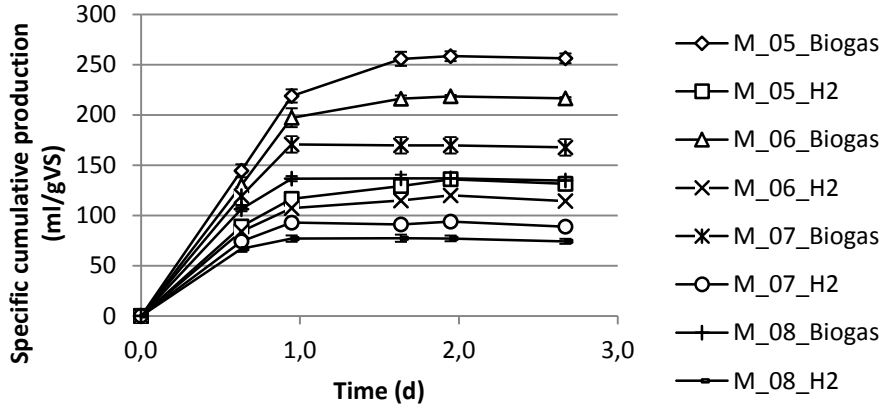

Figure 1.4 Biogas and H_2 production of Mix 5-8 using anaerobic sludge.

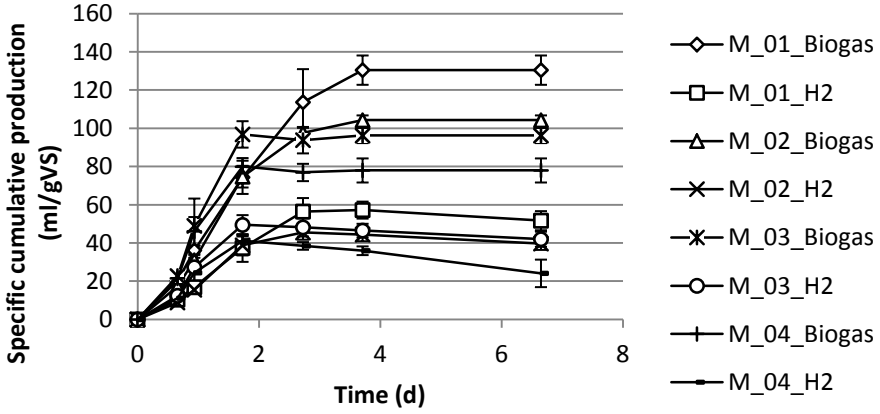

Figure 1.5 Biogas and H_2 production of Mix 1-4 using granular sludge.

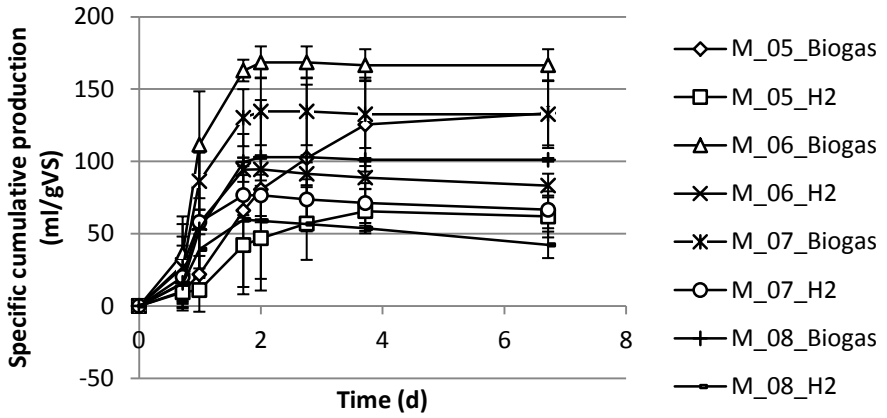

Figure 1.6 Biogas and H_2 production of Mix 5-8 using granular sludge.

Annex 2

Calculation made to compare the hydrogen production of the mixtures to the sum of the hydrogen production of the single sub-fractions that compose them.
Data about DOC, NH_4^+ and VFAs concentrations at the end of BHP tests.

Table 2.1 Comparison between experimental and calculated H_2 yields of 8-mixtures.

	Anaerobic sludge			Granular sludge		
	H_2 real yields (Nml/gVS)	**H_2 estimated yields (Nml/gVS)**	**% error**	**H_2 real yields (Nml/gVS)**	**H_2 estimated yields (Nml/gVS)**	**% error**
MIX 1	129	130	-1%	57	104	-45%
MIX 2	113	110	3%	46	88	-48%
MIX 3	94	89	6%	49	74	-33%
MIX 4	70	69	1%	41	58	-29%
MIX 5	136	130	4%	66	105	-37%
MIX 6	120	110	8%	94	87	8%
MIX 7	94	90	4%	77	72	7%
MIX 8	77	70	10%	60	56	7%

Table 2.2 DOC and Nitrogen concentrations at the end of BHP tests utilizing anaerobic sludge.

Substrate	**DOC (mg/l)**	**N (mg/l)**
Meat, Fish and Cheese	1100 ± 30	441 ± 12
Fruit	1670 ± 17	134 ± 6
Vegetable	1497 ± 6	188 ± 3
Bread and Pasta	1887 ± 55	168 ± 0
MIX 1	1167 ± 21	121 ± 3
MIX 2	987 ± 13	127 ± 3
MIX 3	863 ± 10	125 ± 3
MIX 4	743 ± 29	142 ± 9
MIX 5	1087 ± 57	119 ± 3
MIX 6	968 ± 31	140 ± 6
MIX 7	869 ± 2	157 ± 6
MIX 8	781 ± 23	190 ± 0
Blank test	90 ± 50	152 ± 33

Table 2.3 DOC and Nitrogen concentrations at the end of BHP tests utilizing granular sludge.

Substrate	DOC (mg/l)	N (mg/l)
Meat, Fish and Cheese	1007 ± 29	446 ± 3
Fruit	1640 ± 30	134 ± 10
Vegetable	1503 ± 45	177 ± 6
Bread and Pasta	1873 ± 25	179 ± 0
MIX 1	1197 ± 25	153 ± 9
MIX 2	1027 ± 55	161 ± 3
MIX 3	911 ± 21	177 ± 3
MIX 4	788 ± 33	175 ± 9
MIX 5	1340 ± 80	194 ± 7
MIX 6	1227 ± 38	211 ± 14
MIX 7	1190 ± 95	235 ± 11
MIX 8	1160 ± 61	276 ± 6
Blank test	177 ± 89	184 ± 22

Table 2.4 VFAs concentrations at the end of BHP tests utilizing anaerobic sludge.

VFAs (mg/l)	Meat, Fish and Cheese	Fruit	Vegetable	Bread and Pasta	Blank test
Acetic acid	790 ± 82	1040 ± 129	1163 ± 141	1098 ± 84	136 ± 26
Propionic acid	170 ± 38	27± 12	31 ± 4	19 ± 3	31 ± 10
Isobutyric acid	82 ± 10	13 ± 3	26 ± 2	17 ± 2	17 ± 8
Butyric acid	415 ± 27	1515 ± 160	1037 ± 179	1354 ± 48	15 ± 6
Isovaleric acid	160 ± 7	<10	17 ± 2	23 ± 1	24 ± 13
Valeric acid	<10	<10	<10	<10	17 ± 10
Isocaproic acid	24 ± 7	<10	18 ± 1	20 ± 5	26 ± 15
Caproic acid	53 ± 9	60 ± 20	31 ± 2	80 ± 65	39 ± 6
Heptanoic acid	29 ± 10	46 ± 16	19 ± 7	23 ± 7	71 ± 33

Table 2.5 VFAs concentrations at the end of BHP tests utilizing granular sludge.

VFAs (mg/l)	Meat, Fish and Cheese	Fruit	Vegetable	Bread and Pasta	Blank test
Acetic acid	605 ± 117	525 ± 85	1008 ± 196	1219 ± 118	61
Propionic acid	286 ± 36	101 ± 8	37 ± 4	31 ± 6	18
Isobutyric acid	84 ± 16	21 ± 14	20 ± 4	22 ± 4	10
Butyric acid	328 ± 45	1676 ± 101	1194 ± 84	1323 ± 134	12
Isovaleric acid	152 ± 24	18 ± 9	26 ± 5	31 ± 7	14
Valeric acid	30 ± 7	<10	<10	<10	4
Isocaproic acid	30 ± 7	<15	13 ± 0,8	14 ± 1	6
Caproic acid	44 ± 8	48 ± 32	117 ± 17	109 ± 19	<1,5
Heptanoic acid	23 ± 11	16 ± 4	15 ± 2	16 ± 3	<1,5

Table 2.6 VFAs concentrations at the end of BHP tests utilizing anaerobic sludge.

VFAs (mg/l)	MIX 1	MIX 2	MIX 3	MIX 4	MIX 5	MIX 6	MIX 7	MIX 8	Blank test
Acetic acid	637 ± 76	577 ± 10	528 ± 71	565 ± 73	702±147	521 ± 23	562 ± 38	586 ±148	31 ± 16
Propionic acid	21 ± 9	17 ± 6	12 ± 2	16 ± 2	35 ± 7	43 ± 6	30 ± 17	43 ± 12	4 ± 2
Isobutyric acid	15 ± 1	16 ± 0,6	16 ± 0,8	16 ± 3	20 ± 0,9	19 ± 1	18 ± 2	17 ± 0,9	4 ± 2
Butyric acid	731 ± 26	610 ± 12	510 ± 7	454 ± 64	855±160	621 ± 67	549 ± 48	434± 139	2 ± 1
Isovaleric acid	15 ± 1	13 ± 1	12 ± 2	20 ± 5	<10	<10	10 ± 0,1	20 ± 7	4 ± 2
Valeric acid	<10	<10	<10	<10	<10	<10	<10	<10	1 ± 0,3
Isocaproic acid	16 ± 7	<15	<15	<15	<15	<15	<15	<15	2 ± 0,6
Caproic acid	32 ± 13	33 ± 4	20 ± 8	19 ± 5	16 ± 3	<15	34 ± 17	<15	13 ± 22
Heptanoic acid	19 ± 7	<15	<15	24 ± 15	50 ± 61	15 ± 0,5	15 ± 0,9	33 ± 32	2 ± 1

Table 2.7 VFAs concentrations at the end of BHP tests utilizing granular sludge.

VFAs (mg/l)	MIX 1	MIX 2	MIX 3	MIX 4	MIX 5	MIX 6	MIX 7	MIX 8	Blank test
Acetic acid	594 ± 85	520 ± 53	513 ± 66	406 ± 14	512±149	517 ± 32	553 ± 85	573 ± 42	106 ± 64
Propionic acid	22 ± 10	10 ± 1	20 ± 4	19 ± 2	29 ± 6	42 ± 0,6	52 ± 7	63 ± 18	21 ± 19
Isobutyric acid	17 ± 1	17 ± 0,9	15 ± 0,2	16 ± 0,2	26 ± 3	30 ± 2	33 ± 2	41 ± 0,8	13 ± 13
Butyric acid	701 ± 26	600 ± 24	556 ± 67	423 ± 39	722±206	765 ± 6	711 ± 37	682 ± 10	15 ± 10
Isovaleric acid	18 ± 0,3	18 ± 0,9	26 ± 4	21 ± 0,6	42 ± 6	48 ± 4	54 ± 4	71 ± 4	17 ± 11
Valeric acid	<10	<10	<10	<10	<10	<10	<10	<10	5 ± 5
Isocaproic acid	<15	<15	<15	<15	<15	<15	14 ± 1	18 ± 4	9 ± 9
Caproic acid	204 ± 31	140 ± 23	131 ± 71	93 ± 26	147 ± 70	40 ± 20	29 ± 8	29 ± 20	<1,5
Heptanoic acid	33 ± 16	46 ± 41	29 ± 25	28 ± 13	<15	31 ± 20	34 ± 33	<15	<1,5